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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Frequent alterations of AKT2 oncogene have been frequently detected in human malignancies, including breast carcinoma (1, 2). To better understand the role of AKT2 in breast carcinogenesis, we have isolated a novel protein, APBP, that binds to and is phosphorylated by AKT2. Recent studies showed that p21-activated kinase-1 (PAK1) is activated by AKT2 and that the activated PAK1 induces BAD phopshorylation at Ser112 and Ser136. However, the mechanism by which AKT2 activates PAK1 is unknown. We have recently demonstrated that APBP mediates AKT2 activation of PAK1 by directly binding to PAK1. We have mapped the binding site between APBP and PAK1, i.e., the first and third SH3 domains of APBP interact with a proline-rich region of PAK1. Furthermore, we have shown that APBP protects cells from apoptosis induced by DNA damage and expression of proapoptotic protein Bad. In addition, we have demonstrated that AKT2 is activated by cellular stress and TNFα in human epithelial cells. The activated AKT2 inhibits both JNK and p38 stress kinase activation and protects cells from UV, Heat shock and TNFα-induced apoptosis. We have also demonstrated that AKT2-inhibited JNK1 activation is mediated by AKT2-induced NFκB.

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Introduction

The purpose of this project is to: 1) Examine the incidence and clinical significance of AKT2 alterations in breast cancer, 2) Define the functional interaction between AKT2 and APBP and role of AKT2/APBP in mammary epithelial cell transformation and 3) Determine the FTIs as an inhibitor of AKT2 pathway for breast cancer intervention.

Body:

During the last budget year, we have mainly focused on the functional interaction between APBP and AKT2 and the downstream target of APBP (Aim II).

1. AKT2 interaction protein APBP mediates AKT2 survival signal through activation of PAK1.

. It has been shown that AKT2 exerts its cell survival through induction of p21-activated kinase-1 (PAK1) activity and direct (AKT2-Bad) and indirect (AKT2-PAK1-Bad) phosphorylation of Bad (3-5). However, the mechanism by which AKT2 activates PAK1 is currently unclear. Using yeast two-hybrid system, we have identified an AKT2 interaction protein, APBP. Sequence analyses revealed that APBP possesses four AKT2 phosphorylation consensus sites (RXRXXS/T) and three SH3 domains. APBP coimmunoprecipitates with AKT2. C-terminal regulatory region of AKT2 and the first two SH3 domains of APBP are required for their interaction. AKT2 phosphorylates APBP *in vitro* and *in vivo*. Expression of constitutively activated AKT2 and APBP or APBP alone induces PAK1 activity (Fig. 1). APBP alone is enough to protect cells from apoptosis induced by DNA damage reagent and ectopic expression of Bad (Fig. 2). However, APBP-4A mutant, prepared by converting four AKT2 phosphorylation sites to alanines, attenuated constitutively activated AKT2-induced PAK1 activation and partially abolished AKT2 phosphorylation of Bad (Fig. 2). These data indicate that APBP, an AKT2 substrate, mediates AKT2-induced PAK1 activation and Bad phosphorylation, and thus may play a pivotal role in AKT2 survival pathway.

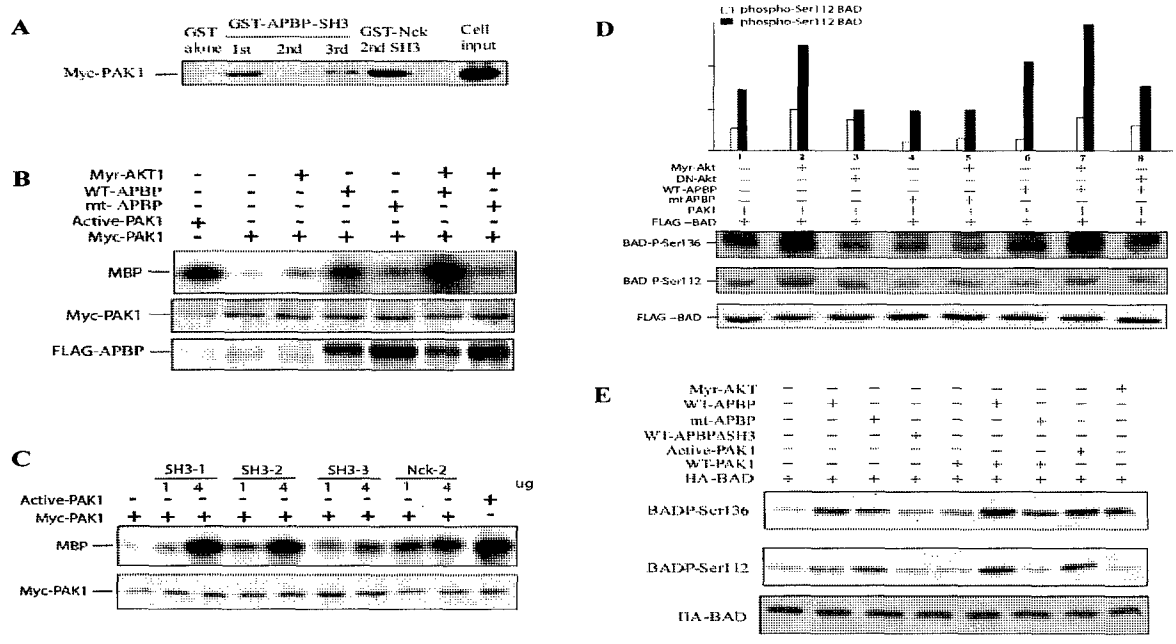

FIG 1

Fig 1. APBPactivates PAK1 and induces BAD phosphorylation at Ser112 and Ser136. *(A) Using GST fused SH3 of APBPto pull down Myc-PAK1 transfected cos7 cell lysates. GST and GST-Nck are used for the negative and positive control. (B) Transfection Myc-PAK1 with active AKT2 and/or WT- APBPor mt- APαB. Immunoprecipitated PAK1, using anti-Myc antibody, is incubated with MBP as substrate. Lower panels show PAK1 and APBPprotein expression using anti-Myc and anti-FLAG antibodies. (C) Different doses (1 or 4 ug) of three GST-fusion SH3 domains of APBPis used to incubate with myc-PAK1 and MBP with radioactive ATP and run the SDS-PAGE. Low panel show protein level of PAK1. PAK1 cotransfection with FLAG-BAD (D) or HA-BAD (E) and AKT2 and WT- APBPor mt- APαB. Cell lysates are immunoprecipitated with anti-FLAG (D) or (E) and run SDS-PAGE, probe filters with anti-S112 and anti-S136 BAD phosphorylation antibodies. Anti-FLAG or anti-HA antibodies are used to detect FLAG-tagged or HA-tagged BAD.*

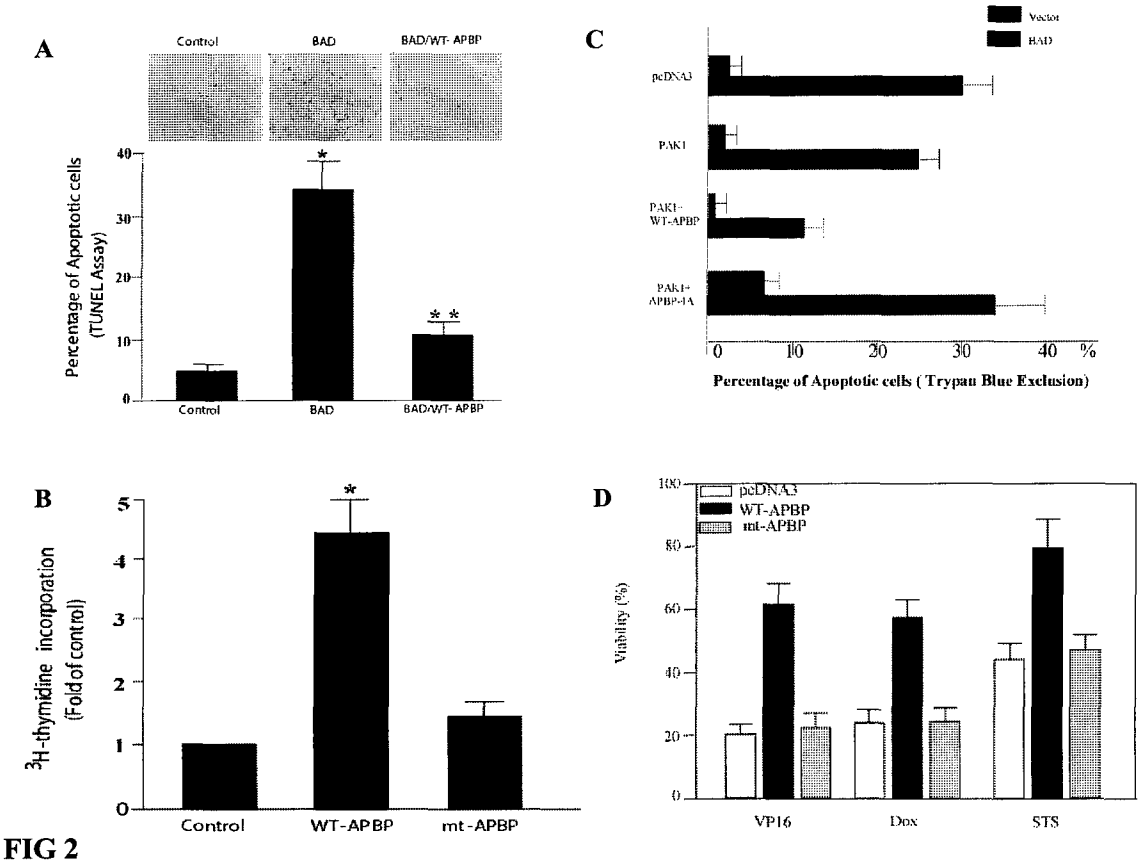

FIG 2

Fig 2. APBPprotects cell from overexpressed-BAD or drugs-induced cell death. *(A) Overexpress BAD with or without APBPin HEK293 cells. The cells were serum starvated for 24 hour and TUNEL assay was used to analyse cell death. (B) Co-transfection APBPand PAK1 with or without BAD in HEK293 cells and trypan blue exclusion is used to assay after 24-hour serum starvation. (C) ^{3}H incorporation was used to measure DNA synthesis in WT- APBPor mt-APBPstably transfected HeLa cells. (D) MTS assay was used to measure cell viability in WT-APBPand mt-APBPstable transfected HeLa cells treated with VP16, Doxorubicin or Staurosporine. All the experiments are repeated three times.*

2. AKT2 is activated by cellular stress- and TNFα and the activated AKT2 inhibits JNK and p38 activities through activation of the NFκB pathway in human epithelial cells. Previous studies have demonstrated that AKT1 and AKT3 are activated by heat shock and oxidative stress via both phosphatidylinositol 3-kinase (PI3K)-dependent and -independent pathways (6). However, the activation and role of AKT2 in the stress response have not been fully elucidated. In this study, we show that AKT2 in epithelial cells is activated by UV-C irradiation, heat shock, and hyperosmolarity, as well as by tumor necrosis factor α (TNFα) through PI3K-dependent pathway. The activation of AKT2 inhibits UV- and TNFα–induced c-Jun N-terminal kinase (JNK) and p38 activities that have been shown to be required for stress- and TNFα-induced programmed cell death (7, 8). Moreover, AKT2 interacts with and phosphorylates IKKα. The phosphorylation of IKKα and activation of NFκB mediates AKT2 inhibition of JNK but not p38. Furthermore, PI3K inhibitor or dominant negative AKT2 significantly enhances UV- and TNFα-induced apoptosis, whereas expression of constitutively active AKT2 inhibits programmed cell death in response to UV and TNFα stimulation with accompanying decreased JNK and p38 activity. These results indicate that activated AKT2 protects epithelial cells from stress- and TNFα-induced apoptosis by inhibition of stress kinases and provide the first evidence that Akt inhibits stress kinase JNK through activation of NFκB pathway. This work has recently been published in a *J. Biol. Chem.* 2002 June 4 [epub ahead of print].

Key Research Accomplishment

1 APBP is a downstream of AKT2 and mediates AKT2 survival signaling through interaction and activation of PAK1.

2 AKT2 inhibits JNK and p38 activation and prevents stress-induced apoptosis through activating NFκB Pathway. As JNK and p38 activation is required for chemotherapeutic drug-induced programmed cell death, activation and overexpression of AKT2 in human breast cancer will contribute to chemoresistance. Therefore, development reagent(s) to target AKT2 will greatly benefit to breast cancer intervention.

Reportable Outcomes

1. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-OH kinase/AKT2 pathway in human ovarian cancer. *Oncogene* 19, 2324-2330, 2000
2. Phosphoinositide-OH kinase/AKT2, activated in breast cancer, regulates and is induced by Estrogen Receptor α (ERα) via interaction between ERα and PI3K. *Cancer Res.* 61,5985-5991, 2001 (see Appendix).
3. Akt/PKB Binding Protein, APαB, Mediates Akt Survival Signal through Activation p21-activated Kinase. *Oral presentation in Oncogene Meeting* (17th Meeting on Oncogene Meeting, Jun 20-23, 2001, Hood College, Frederich, Maryland).
4. Inhibition of JNK by cellular stress- and TNFα-induced AKT2 through activation of NFκB pathway in human epithelial cells. [*J. Biol. Chem.* 2002 June 4 (epub ahead of print), see Appendix)

Conclusion

1. Activated AKT2 protects epithelial cells from stress- and TNFα-induced apoptosis by inhibition of stress kinases JNK and p38 through activation of NFκB pathway. This work provided evidence to target AKT2 for overcome drug resistance in breast cancer which proposed in the Aim III.
2. APBP mediates AKT2/PAK1-induced BAD phosphorylation in cell survival signaling pathway, implicating APBP as a potential target for breast cancer intervention.

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Appendices

1. Yuan, Z.Q., Sun, M., Feldman, R.I., Wang, G., Ma, X., Coppola, D., Nicosia, S.V. and Cheng, J.Q. Frequent alterations of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/AKT2 pathway in human ovarian cancer. Oncogene 19:2324-2330, 2000.
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4. Curriculum Vitae

Oncogene (2000) 19, 2324–2330

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Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer

Zeng Qiang Yuan[1], Mei Sun[1], Richard I Feldman[2], Gen Wang[1], Xiao-ling Ma[1], Chen Jiang[1], Domenico Coppola[1], Santo V Nicosia[1] and Jin Q Cheng*,[1]

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We previously demonstrated that AKT2, a member of protein kinase B family, is activated by a number of growth factors via Ras and PI 3-kinase signaling pathways. Here, we report the frequent activation of AKT2 in human primary ovarian cancer and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase (PI 3-kinase)/Akt pathway. *In vitro* AKT2 kinase assay analyses in 91 ovarian cancer specimens revealed elevated levels of AKT2 activity (>3-fold) in 33 cases (36.3%). The majority of tumors displaying activated AKT2 were high grade and stages III and IV. Immunostaining and Western blot analyses using a phospho-ser-473 Akt antibody that detects the activated form of AKT2 (AKT2 phosphorylated at serine-474) confirmed the frequent activation of AKT2 in ovarian cancer specimens. Phosphorylated AKT2 in tumor specimens localized to the cell membrane and cytoplasm but not the nucleus. To address the mechanism of AKT2 activation, we measured *in vitro* PI 3-kinase activity in 43 ovarian cancer specimens, including the 33 cases displaying elevated AKT2 activation. High levels of PI 3-kinase activity were observed in 20 cases, 15 of which also exhibited AKT2 activation. The remaining five cases displayed elevated AKT1 activation. Among the cases with elevated AKT2, but not PI 3-kinase activity (18 cases), three showed down-regulation of PTEN protein expression. Inhibition of PI 3-kinase/AKT2 by wortmannin or LY294002 induces apoptosis in ovarian cancer cells exhibiting activation of the PI 3-kinase/AKT2 pathway. These findings demonstrate for the first time that activation of AKT2 is a common occurrence in human ovarian cancer and that PI 3-kinase/Akt pathway may be an important target for ovarian cancer intervention. *Oncogene* (2000) **19,** 2324–2330.

Keywords: Akt; PI 3-kinase; signal transduction; ovarian cancer

Akt/PKB represents a subfamily of the serine/threonine protein kinases (Bellacosa *et al.*, 1991; Jones *et al.*, 1991a, b; Cheng *et al.*, 1992; Konishi *et al.*, 1995; Nakatani *et al.*, 1999). Three members of this family, AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ have been identified. Akt is activated by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress (Franke *et al.*, 1995; Burgering *et al.*, 1995; Shaw *et al.*, 1998; Liu *et al.*, 1998). Activation of Akt and AKT2 by growth factor is mediated by PI 3-kinase (Franke *et al.*, 1995; Meier *et al.*, 1997; Liu *et al.*, 1998). Active Ras and Src have also shown to activate Akt and AKT2 and this activation is blocked by wortmannin, a PI 3-kinase inhibitor, indicating that Ras and Src also mediate the activation of Akt and AKT2 and are located upstream of PI 3-kinase (Datta *et al.*, 1996; Liu *et al.*, 1998). Several lines of evidence suggest that PI 3-kinase regulates Akt activation through the following mechanism: the product of PI 3-kinase, phosphatidylinositol-3,4,5-trisphosphate, binds to the pleckstrin homology (PH) domain of Akt after growth factor stimulation, resulting in recruitment of Akt to the cell membrane. A conformational change of Akt follows, which allows residues Thr-308 and Ser-473 to be phosphorylated by upstream kinases, PDK-1 and PDK2 or ILK, respectively (Alessi and Cohen, 1998; Delcommenne *et al.*, 1998). Several downstream targets of Akt, each of which contains the Akt phosphorylation consensus sequence R-X-R-X-X-S/T-F/L, have been identified (Alessi and Cohen, 1998), pointing to the possible mechanisms by which Akt promotes cell survival and blocks apoptosis. One such target is GSK3. Akt phosphorylates GSK3 and leads to inactivation of GSK3, accumulation of β-catenin, and activation of *Myc* transcription. There is also evidences that Akt phosphorylates the proapoptotic proteins BAD, caspase-9 and transcription factor FKHRL1, resulting in reduced binding of BAD to Bcl-X_L and inhibition of caspase-9 protease activity and Fas ligand transcription (Datta *et al.*, 1997; del Peso *et al.*, 1997; Cardone *et al.*, 1998; Brunet *et al.*, 1999). Recent studies demonstrated that PTEN/MMAC1 tumor suppressor and SHIP, tyrosine and inositol phosphatases, dephosphorylate phosphatidylinositol-3,4,5-triphos-phate, thus, inhibiting the PI 3-kinase Akt signaling pathway (Stambolic *et al.*, 1998; Aman *et al.*, 1998).

Several members within the PI 3-kinase/Akt pathway, including PI 3-kinase, PTEN, AKT2, and β-catenin, have been implicated in human neoplasms (Alessi and Cohen, 1998). Overexpression of p85, the regulatory subunit, or p110, the catalytic subunit, of PI 3-kinase is able to transform cells (Chang *et al.*, 1997; Jimenez *et al.*, 1998). Alterations of PI 3-kinase have also been detected in a number of human malignancies (Shayesteh *et al.*, 1999; Phillips *et al.*, 1998). Mutation and/or down regulation of the *Pten* are frequently observed in endometrial carcinoma, glioblastoma, breast cancer and prostate carcinoma (Stambolic *et*

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al., 1998). We and others have previously demonstrated alterations of *AKT2* at DNA, mRNA and/or protein level in several types of human malignancy (Cheng *et al.*, 1992, 1996; Bellacosa *et al.*, 1995; Ruggeri *et al.*, 1998). In particular, amplification/overexpression of *AKT2* has been detected in 10–20% of ovarian carcinomas and pancreatic cancers. However, increased activation of AKT2 in human tumors has not been demonstrated previously.

In this report, we show that kinase activity of AKT2 is frequently elevated in human primary ovarian tumor. *In vitro* AKT2 kinase assays were performed in 91 primary human ovarian tumors, including 34 serous cystadenocarcinomas, four mucinous cystadenocarcinomas, 25 papillary serous adenocarcinomas, 10 endometrioid adenocarcinomas, two borderline tumors, five granulosa cell tumors and 11 other rare types of tumors including adenosarcoma, thecoma, fibroma, and mesodermal mixed tumor. Lysates from tumor specimens containing equivalent amounts of protein were precleared and incubated with anti-AKT2 antibody, which specifically reacts with AKT2, in the presence of protein-A : protein-G beads. The immunoprecipitates were subjected to *in vitro* kinase assay using Histone H2B as the substrate. *In vitro* kinase assays were carried out three times for each specimen. Average reading of the kinase activity threefold higher than that in normal ovarian tissue was considered elevated AKT2 activity. The results revealed an elevated level of AKT2 kinase in 33 specimens (36.3%), including 15 serous cystadenocarcinomas (44%), 14 papillary serous adenocarcinomas (56%) and four other types of tumor including one adenosarcoma, two malignant clear cell tumors, and one mixed mullerian tumor. Examples of AKT2 kinase activity in ovarian cancer are shown in Figure 1. Interestingly, the majority of cases exhibiting activation of AKT2 were serous adenocarcinomas (29/33). None of endometrioid, borderline, mucinous, and granulosa cell tumors exhibited elevated AKT2 kinase activity.

To examine whether variations in AKT2 protein expression level contributes to the level of AKT2 activity observed in ovarian tumor specimens, we analysed its expression by Western blot analyses using a specific anti-AKT2 antibody. A high level of AKT2 protein was detected in 42 of 91 cases (46%). The 33 cases that exhibited elevated AKT2 activity displayed variable levels of AKT2 protein, 25 of which expressed AKT2 at high or moderate levels (Figure 1 and Table 1). These results were also confirmed by immunohistochemical staining of the tumor tissue section (data not shown).

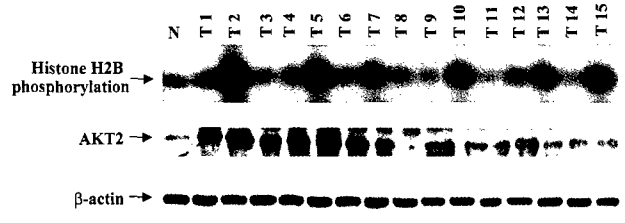

Figure 1 Elevated level of AKT2 kinase activity in ovarian cancer. Top panel: *In vitro* kinase assays of AKT2 immunoprecipitated from 15 frozen representative tumor specimens. Frozen tissues from ovarian carcinoma was mechanically smashed in liquid nitrogen and lysed by a Tissue Tearor in a lysis buffer (Liu *et al.*, 1998). Lysates were incubated with anti-AKT2 antibody in the presence of protein A-protein G (2 : 1) agarose beads for 2 h at 4°C. Following extensive wash, immunoprecipitates were subjected to *in vitro* kinase assay (Liu *et al.*, 1998). Histone H_2B was used as the exogenous substrate. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Phosphorimager. AKT2 is activated in cases T2, T5, T7, T10, T13, and T15. Middle panel: Western blot analysis of AKT2 expression in ovarian carcinoma. The blot was reprobed with anti-β-actin antibody (Bottom panel)

Previous studies demonstrated that phosphorylation of Thr-308 and Ser-473 of Akt1 is required for its full activation. Upon stimulation of PI 3-kinase by growth factors, Akt1 becomes phosphorylated at these two residues. It has been shown that AKT2 is activated by phosphorylation of the equivalent residues (Thr-309 and Ser-474) (Alessi and Cohen, 1998). The homology of Akt1 to AKT2 and AKT3 is 90.4% and 87.8% at the amino acid level, respectively (Cheng *et al.*, 1992; Nakatani *et al.*, 1999), and the sequence of the serine-473 phosphorylation site of Akt1 is highly conserved in AKT2 and AKT3 (Figure 2a).

Anti-phospho-Ser473 Akt antibody has widely been used to identify Akt1 activation. To determine whether such an antibody can be used to detect AKT2 activation in ovarian cancer specimens by Western blotting and immunostaining, we tested whether a phospho-Ser473 Akt antibody reacted with phosphorylated AKT2. HA-AKT2 expression construct was transfected to COS-7 cells. After serum-starvation overnight and IGF-1 stimulation for 10 min, HA-AKT2 was immunoprecipitated with anti-HA monoclonal antibody. The immunoprecipitates were separated by SDS–PAGE and probed with phospho-Ser473 Akt antibody. The results showed that the antibody against phospho-Ser473 Akt strongly reacted with phospho-Ser 474 of AKT2 (Figure 2b).

We next examined AKT2 phosphorylation by Western blot analyses of AKT2 immunoprecipitates with phospho-Ser473 Akt antibody in 43 ovarian tumor specimens including the 33 cases with and 10 cases without elevated AKT2 kinase activity. A sheep anti-AKT2 antibody was used to incubate with equal amount of protein from ovarian tumor lysates in the presence of protein-A and protein-G agarose. After extensive wash with the lysis buffer, the AKT2 immunoprecipitates were separated by SDS–PAGE and the blots were probed with the rabbit polyclonal phospho-Ser473 Akt antibody (New England Biolab). Phosphorylated AKT2 was detected only in ovarian tumors with elevated AKT2 kinase activity; for example, see data from tumors T2, 5, 7, 10 and 13, which exhibit high AKT2 kinase activity, shown in Figure 2c. These data confirm the results obtained from *in vitro* AKT2 kinase assay and indicate that AKT2 activity is regulated by phosphorylation at Ser474 in human primary tumors.

Table 1 AKT2 activation and clinical stage

		AKT2 kinase activity		*AKT2 protein level*	
Stage	n	*Normal*	*High*	*Low*	*High/moderate*
I	7	7	0	5	2
II	11	11	0	6	5
IIIA	5	5	0	1	4
IIIC	53	27	26	31	22
IV	15	8	7	6	9

Due to the fact that stromal tissues approximately account for 20~30% of the tumor specimens used in this study, we examined whether activation of AKT2 is derived from tumor cells or stromal tissues by immunostaining the tumor paraffin sections with phospho-Ser473 Akt antibody. We first tested if the phospho-Ser473 Akt antibody is capable of recognizing phosphorylated AKT2 by immunohistochemistry. Cell paraffin blocks were prepared from serum-starved and serum-starved EGF-stimulated OVCAR-3 cells that overexpress AKT2 (Cheng *et al*., 1992). Phospho-Ser473 antibody was used to detect phosphorylated AKT2 in the section from these blocks by immunohistochemical means. Strong positive staining was observed in EGF-stimulated but not serum-starved OVCAR-3 cells (panel 1~2 in Figure 3a). Phosphorylation status of AKT2 in these cells was confirmed by Western blot analysis with phospho-S473 Akt antibody (lanes 1 and 2 in Figure 3b).

Immunostaining of the tumor paraffin sections with phospho-Ser473 antibody was performed in 43 ovarian cancer specimens including 33 cases exhibiting elevated AKT2 kinase activity and 10 cases without AKT2 activation. Positive staining of tumor cells was observed in 38 cases including 33 with and five without AKT2 activation detected by *in vitro* kinase assay and Western blot analyses. The fact that phospho-Ser473 Akt antibody recognizes Akt1, AKT2 and possible AKT3 suggests that activation of AKT1 or AKT3 present in these five cases, which may be due to different expression levels of three isoforms of Akt. Immunohistochemical staining of paraffin tissue sections with anti-AKT1 and -AKT2 antibodies revealed that AKT1 but not AKT2 is highly expressed in these five specimens (Figure 3c). Moreover, *in vitro* kinase assay showed that AKT1 kinase activity is elevated in these specimens (Figure 3d, and also see case 8 in Figures 1 and 4). Unfortunately, no good AKT3 antibody is commercially available at present time, therefore, we could not determine if AKT3 protein is altered in these specimens. Strikingly, phosphorylated Akt localizes to cytoplasm and cell membrane. Even in EGF-stimulated OVCAR-3 cells, activated Akt stays in cytoplasm and cell membrane. No nuclear staining was observed (panels 2 and 4 of Figure 3a).

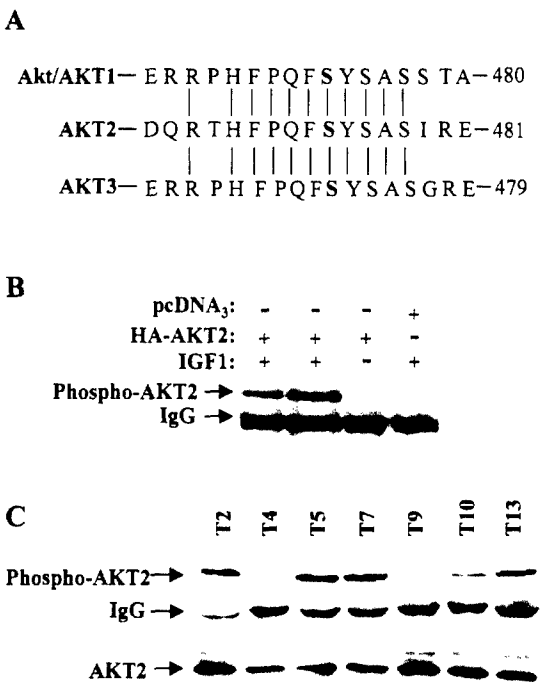

Figure 2 Phospho-Akt-Ser473 antibody recognizes phosphorylation form of AKT2 that is detected in ovarian cancer. (**a**) Comparison of C-terminal amino acid sequence of AKT1 and AKT2 and AKT3. Phosphorylation of serine-473 (AKT1) is well conserved in AKT2 (Ser-474) and AKT3 (Ser-472). (**b**) Western blot analysis of HA-AKT2 immunoprecipitates from COS-7 cells transfected with pcDNA$_3$-HA-AKT2 or pcDNA$_3$ vector alone. After transfection, the cells were serum-starved overnight and stimulated with IGF-1 for 10 min (lanes 1 and 2 from left) prior to harvesting cells. Immunoprecipitation was carried out with anti-HA monoclonal antibody and separated by SDS PAGE. The blot was detected with Phospho-Akt-Ser473 antibody. (**c**) Western blot analysis of phosphorylation of AKT2 in ovarian cancer specimens. The tumor lysates were incubated with anti-AKT2 antibody. The resulting immunoprecipitates were separated by SDS PAGE and detected with polyclonal phospho-Ser473 Akt antibody (upper panel) or polyclonal anti-AKT2 antibody (lower panel)

One mechanism that could result in an increased activation of AKT2 is an up-regulation of PI 3-kinase. PI 3-kinase is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit. Three isoforms of p85 and p110 have been cloned, namely p85α, p85β, p85γ, p110α, p110β and p100γ. Recent studies demonstrated transforming activity of p110 and p85 and frequent activation PI 3-kinase in colon and ovarian carcinoma cell lines (Chang *et al*., 1997; Jimenez *et al*., 1998; Shayesteh *et al*., 1999; Phillips *et al*., 1998). To examine PI 3-kinase activity in tumor tissues, immunoprecipitation with a pan-p85 specific antibody was performed in 43 ovarian cancer cases, including 33 with and 10 without AKT2 activation (Klippel *et al*., 1993). Following extensive wash, the immunoprecipitates were subjected to *in vitro* PI 3-kinase assay using L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P$_2$) or L-α-phosphatidylinositol-4-phosphate (PI-4-P$_1$) as substrates. The conversion of PI-4,5-P$_2$ to PI-3,4,5-P$_3$ or PI-4-P$_1$ to PI-3,4-P$_2$ was determined by autoradiography and quantitated by Phosphorimager. Elevated PI 3-kinase activity was detected in 20 ovarian cancer specimens, 15 of which exhibited AKT2 activation and the other five displayed AKT1 activation (Figures 3 and 4). Since p110α has been shown to be overexpressed and activated in ovarian cancer cell lines (Shayesteh *et al*., 1999), we examined whether increased levels of activity observed in our primary ovarian tumor specimens resulted from increased levels of p110 expression. PI 3-kinase was immunoprecipitated with an anti-p85 antibody from equal amounts of tumor extract protein. The immunoprecipitates were then separated on a SDS PAGE, transferred to a membrane and probed with a specific anti-p110α antibody. Figure 4 shows a correlation between p110α expression levels and PI 3-kinase activity in tumor samples. Quantitative analysis by a BioImager (Genomic Solutions Inc.) revealed that the levels of p110α expression in the tumors with PI 3-kinase activation are 2~4-folds higher than that in the specimens showing no elevated PI 3-kinase, except T12 that exhibited high level of p110α protein but no significant PI 3-kinase activation. These data indicate that increases of PI 3-kinase activity contribute to AKT activation in human primary ovarian carcinoma.

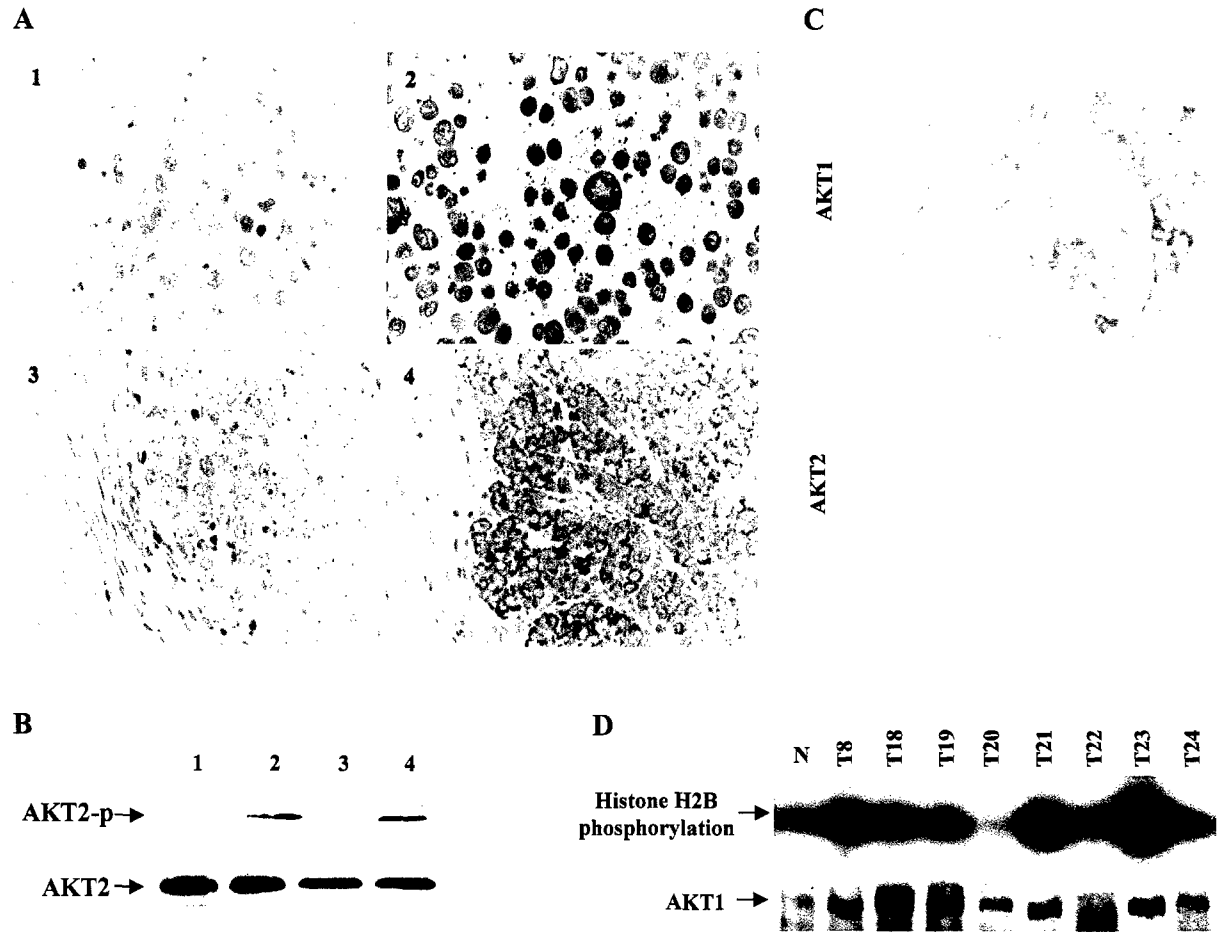

Figure 3 Phosphorylated Akt detected by immunostaining localizes to cell membrane and cytoplasm of ovarian tumor cells. The paraffin sections were subjected to antigen retrieval by boiling in a microwave and then incubated in a blocking solution and an avidin/biotin blocking kit (Vector). The primary antibody to phospho-S473 Akt (Upstate Biotechnology) was applied at a dilution of 1:200. After incubation, the slides were treated with biotinylated rabbit anti-goat immunoglobulin and streptavidin and biotinylated alkaline phosphatase. (**a**) Sections of paraffin blocks prepared from serum-starved (1), EGF-stimulated OVCAR3 cells (2) and 2 primary ovarian carcinoma specimens (3~4) were stained with phospho-Ser473 antibody. No staining was observed in serum starved OVCAR3 cells and a specimen without elevated AKT2 kinase activity (1 and 3), whereas strong reaction with phospho-Ser473 antibody is seen in EGF-stimulated OVCAR3 cells and a tumor specimen exhibiting AKT2 activation. (**b**) Western blot analysis of the AKT2 immunoprecipitates from the cells and the tissues that are used for immunostaining in Figure 3a. The filter was detected with phospho-Ser473 antibody (upper panel) and anti-AKT2 antibody (lower panel). (**c**) Immunohistochemical staining of an ovarian cancer specimen with anti-AKT1, and -AKT2 antibodies. (**d**) *In vitro* kinase assay (upper panel) and Western blot (bottom panel) of AKT1 immunoprecipitates from frozen tumor specimens. Results represent three independent experiments. AKT1 is activated in cases T8, T18, T19, T21, and T23

Another mechanism shown to result in activation of Akt is inactivation or loss of expression of PTEN. Inactivating mutations of the *Pten* tumor suppressor gene, on chromosome 10q23, have been described in prostate, endometrial and ovarian endometrioid carcinomas (Obata *et al*., 1998). Previous studies have also observed down-regulation of PTEN protein in prostate cancer (Wu *et al*., 1998; McMenamin *et al*., 1999). Therefore, we examined whether down-regulation of PTEN is associated with AKT2 activation in ovarian cancer. We analysed PTEN expression in 18 ovarian cancer specimens displaying elevated AKT2 activity by Western blot. A large reduction in PTEN protein expression was observed in three cases, two of which were serous cystadenocarcinomas and one was a papillary serous adenocarcinoma (Figure 5). In these cases, we did not detect either mutations or low mRNA level of the *Pten* gene (data not shown). Interestingly, we failed to observe elevated AKT2 kinase activity in 10 endometrioid adenocarcinomas examined, even though PTEN mutations have most frequently been detected in this type of ovarian tumor. These results suggest that down-regulation of PTEN protein expression, either by translational or post-translational changes, may be a factor in elevating AKT2 activation in non-endometrioid ovarian cancer.

We next examined the relationships between activation of AKT2 and tumor stage and grade. The results are presented in Tables 1 and 2. High levels of AKT2 activity were seen in stages III and IV (33/68, 48.5%) but not stages I and II. The incidence of AKT2 activation increased with increasing grade. High levels of AKT2 kinase activity were detected in 52.9% of grades III and IV tumors, whereas activation of AKT2 was observed in only 15% of grades I and II tumors. These data indicate that activation of AKT2 in ovarian carcinoma is associated with late stage and high-grade tumors, and suggest an increase in the activation of AKT2 kinase as ovarian cancer progresses to a more aggressive phenotype.

It has been shown that Akt induces cell survival and suppresses the apoptotic death of a number of cell types induced by a variety of stimuli, including growth factor

Figure 4 Activation of PI 3-kinase in human ovarian cancer specimens. *In vitro* PI 3-kinase assay of the anti-p85 immunoprecipitates from 12 frozen ovarian cancer specimens. The tumor tissue lysates were immunoprecipitated with pan-p85 antibody (Santa Cruz). Following wash, the presence of PI 3-kinase activity in immunoprecipitates was determined by incubating the beads with reaction buffer. Phospholipids were extracted and separated by thin-layer chromatography as previously described (Jiang *et al*., 2000). The conversion of PI-4,5-P_2 to PI 3-phosphate and PI-4-PI to PI-3,4-P_2 was determined by autoradiography and quantitated by using a Phosphorimager. Elevated levels of PI 3-kinase activity (top panel) and p110α protein (middle panel) are detected in cases 2, 7, 8, 12, and 13. The expression levels of p110α are quantified by a BioImager. The density of each band is indicated at the bottom

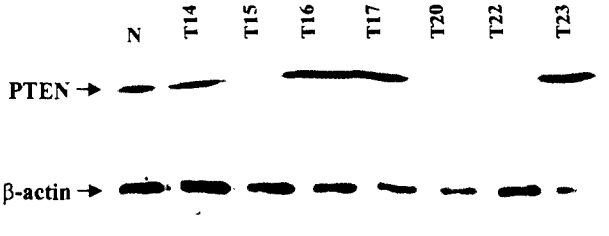

Figure 5 Down regulation of PTEN in human ovarian carcinoma. Western blot analyses of the tumor tissue lysates with anti PTEN (upper) and β-actin (bottom) antibody. No detectable level of PTEN was observed in three cases

Table 2 AKT2 activation and grade

		AKT2 kinase activity	
Grade	n	*Normal*	*High*
1	21	17	4
2	19	17	2
3	49	24	25
4	2	0	2

withdrawal, cell-cycle discordance and loss of cell adhesion. To assess the influence of PI 3-kinase/AKT activation on the cell growth of human ovarian cancer cells, we have performed *in vitro* kinase assay and observed activation of AKT2 and PI 3-kinase in 2 of 5 ovarian cancer cell lines (Figure 6a). If PI 3-kinase/Akt pathway is crucial for survival in such ovarian tumor cells, blocking the activity of PI 3-kinase/Akt is expected to inhibit the cell growth and/or induce apoptosis. To test this hypothesis, three ovarian cancer cell lines with or without PI 3-kinase/AKT2 activation were treated with PI 3-kinase inhibitors, wortmannin (200 nM) or LY294002 (40 μM), or vehicle (DMSO), for 12 h in a medium containing 1% fetal calf serum. Those cell lines exhibiting elevated levels of PI 3-kinase and AKT2 activity underwent apoptosis after treatment with wortmannin or LY294002, whereas no apoptosis was detected in the cell line (e.g., A2780) without PI 3-kinase/AKT2 activation (Figure 6).

In this study, we have demonstrated frequent activation of AKT2 kinase in human primary ovarian cancer by *in vitro* kinase assay. Western blot and immunohistochemical staining. Phosphorylated AKT2 has a cell membrane and cytoplasmic but not nuclear localization. Increased PI 3-kinase activation is observed in the majority of cases displaying AKT2 activation. High levels of AKT2 protein expression are also frequently detected in ovarian cancer specimens. Down regulation of PTEN was observed in non-endometrioid ovarian tumors, which may contribute to AKT2 activation. Moreover, activation of AKT2 is associated with high grade and late stage ovarian cancer and direct inhibition of PI 3-kinase Akt pathway induces apoptosis in ovarian cancer cell lines exhibiting activation of PI 3-kinase and AKT2.

Previous studies showed that alteration of oncogene could occur at DNA, mRNA, protein, or enzymatic level. An extensive review of the topic indicates that the frequency of oncogene amplification in primary tumors is about 5–38%, depending on tumor type, stage, and grade as well as the individual gene (Brison, 1993). The incidence of overexpression of some oncogenes in certain tumors is even higher due to the mechanism of enhanced transcription or translation, and mRNA or protein stabilization (Brison, 1993; Devilee and Cornelisse, 1994; Berns *et al*., 1995). We and others have previously shown alterations of *AKT2* at DNA and mRNA levels in ~12% and ~25% of ovarian carcinoma, respectively (Cheng *et al*., 1992; Bellacosa *et al*., 1995). In this study, we demonstrate activation of AKT2 kinase in 36.3% of primary ovarian tumors with highest frequency (49%) in serous adenocarcinomas. Moreover, we have also observed overexpression of AKT2 protein in 46% of ovarian cancer specimens examined. These data, as compared to *AKT2* alteration at DNA and RNA levels, indicate that activation and overexpression of AKT2 protein by aberrant translational regulation and posttranslational modification of AKT2 is a more common occurrence in human ovarian cancer. The fact that overexpression of wild type AKT2 in NIH3T3 cells results in malignant transformation (Cheng *et al*., 1997) suggests that activation and overexpression of AKT2 in human ovarian carcinoma could play an important role in the development of this malignancy.

Several studies demonstrated that ectopically expressed Akt1 and AKT2, following growth factor stimulation, initially locates to cell membrane and then translocates to the nucleus (Andjelkovic *et al*., 1997; Meier *et al*., 1997). This is thought to allow Akt mediated phosphorylation of nuclear transcription factors, such as Forkhead proteins and CREB (Brunet *et al*., 1999; Kops *et al*., 1999; Du and Montminy, 1998). In contrast, in primary ovarian tumor specimens examined, we found that phosphorylated AKT2 localizes to cell membrane and cytoplasm but not the nucleus. Moreover, activated endogenous AKT2 in AKT2-overexpressing OVCAR cells was also localized to cell membrane and cytoplasm only. These conflicting observations may be due to the fact that ectopic overexpression of Akt affects its subcellular localization.

We and others have previously documented that AKT2 is a downstream target of PI 3-kinase and is activated by a number of growth factors (Meier *et al*.,

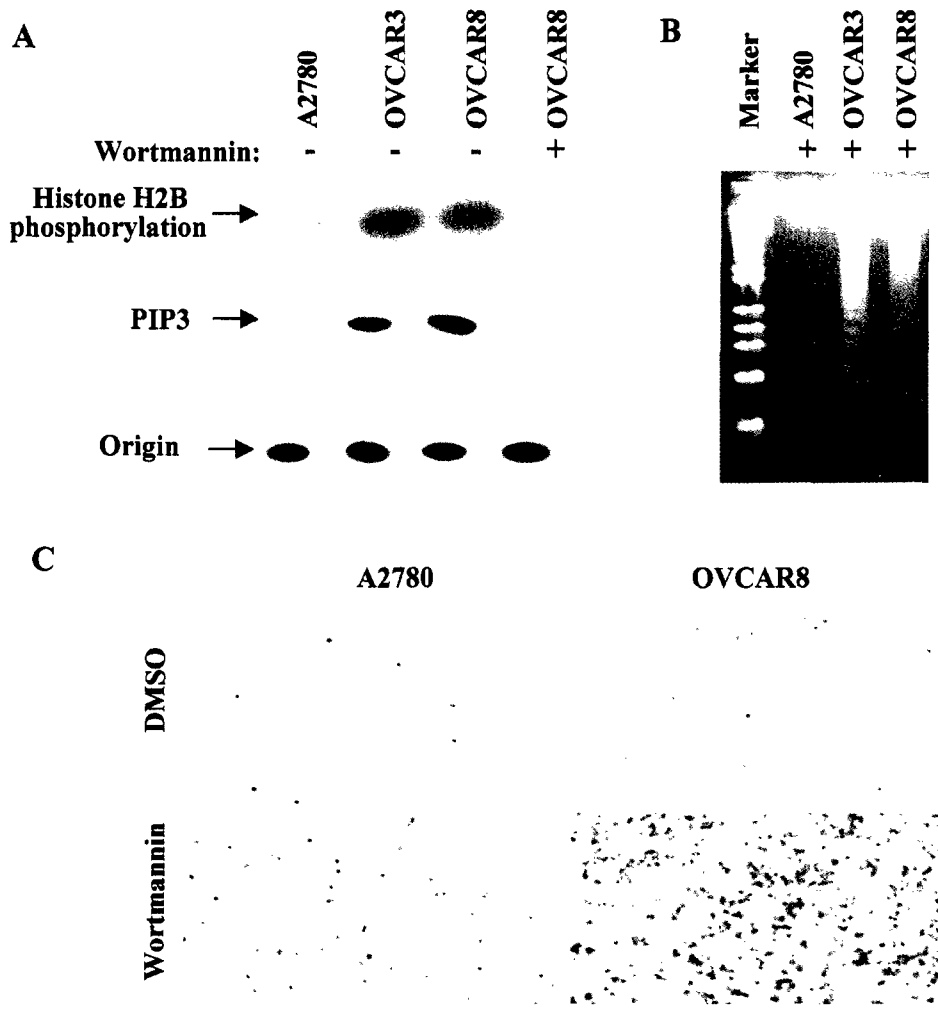

Figure 6 Inhibition of PI 3-kinase/AKT2 activity induces apoptosis in PI 3-kinase/AKT2-activating ovarian cancer cell lines. (**a**) *In vitro* Akt and PI 3-kinase assay of the immunoprecipitates from A2780, OVCAR3 and OVCAR8 cells. AKT2 (upper panel) and PI 3-kinase (bottom panel) are activated in OVCAR3 and OVCAR8 cells. (**b**) and (**c**) DNA fragmentation and Tunel assay showing that Wortmannin blocks activity of PI 3-kinase and AKT2, and induces apoptosis in OVCAR3 and OVCAR8 cells. The cells were seeded into 60 mm dishes and grown for 24 h and then treated with wortmannin (200 nM) or LY294002 (40 μM) for 12 h. Apoptosis was determined by TdT-mediated dUTP nick end labeling (Tunel) using an *in situ* cell death detection kit (Boehringer). To detect DNA fragmentation, cellular DNA was prepared using the DNA kit (Qiagen). The DNA was analysed on 1.5% agarose gel and visualized by ethidium bromide staining

1997; Liu *et al*., 1998). Active Ras and Src significantly induce AKT2 activation (Liu *et al*., 1998). Recent studies have shown that Akt activity is regulated by *PTEN*, which reduces intracellular levels of PI-3,4,5,-P_3 in cells by converting PI-3,4,5-P_3 to PI-4,5-P_2 and, thus, inhibits PI 3-kinase/Akt signaling pathway (Stambolic *et al*, 1998; Aman *et al*., 1998). Therefore, AKT2 activation in human ovarian cancer may result from; (a) PI 3-kinase activation; (b) *Pten* mutation; (c) overexpression of AKT2; (d) alterations of growth factor receptor, such as overexpression or mutation of EGFR; (e) *ras* mutation; or (f) active mutation of the *AKT2* gene. In this report, we document that nearly half of cases with AKT2 activation (15/33) display PI 3-kinase activation, which supports a recent observation of PI 3-kinase activation in ovarian cancer cell lines (Shayesteh *et al*., 1999). Down regulation of PTEN was detected in three non-endometrioid cases of ovarian tumors. Interestingly, we did not observe either lack of PTEN expression or AKT2 activation in all 10 endometrioid adenocarcinomas examined, implying that the *Pten* may not be mutated in these specimens. We have also performed single strain conformation polymorphism analyses of the tumors carrying activated AKT2. No *AKT2* mutation was observed. The cases exhibiting AKT2 activation express variable levels of AKT2 protein, suggesting that expression of AKT2 protein is required for activation of AKT2 in ovarian tumor.

The fact that three members of Akt family are down stream targets of PI 3-kinase and are regulated by similar mechanism suggests that AKT1 and AKT3 may also be activated in human ovarian cancer. In this study, we focused on activation of AKT2 in primary ovarian cancer. However, we have shown activation of AKT1 and PI 3-kinase but not AKT2 in five ovarian cancer specimens, which express high levels of AKT1 but very low levels of AKT2 (Figure 3). Additional experiments to evaluate the significance of this finding are in progress.

We have previously demonstrated that an activated Ras significantly activates AKT2. We have also recently documented that farnesyltransferase inhibitor (FTI)-277, an anticancer drug blocking the posttranslational farnesylation of oncogenic Ras, inhibits PI 3-kinase and AKT2 activity and induces apoptosis in

AKT2 overexpressing cell lines (Jiang *et al*., 2000). Overexpression of AKT2, but not Ras, sensitizes NIH3T3 cells to FTI-277. However, FTI-277 is not a direct inhibitor of PI 3-kinase and AKT2. In this study, we demonstrate that direct inhibition of PI 3-kinase/Akt pathway by wortmannin or LY294002 induces apoptosis in ovarian cancer cell lines with PI 3-kinase/AKT2 activation. Moreover, we have also shown that the majority of tumors with activated AKT2 are high grade and late stage. These data indicate that activation of PI 3-kinase/Akt may play a pivotal role in development of human ovarian cancer, especially in tumor progression, and that PI 3-kinase/Akt pathway could be an important target for intervention of this malignancy. Future studies are required to define the role of Akt activation in ovarian malignant transformation.

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Advances in Brief

Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, Activated in Breast Cancer, Regulates and Is Induced by Estrogen Receptor α (ERα) via Interaction between ERα and PI3K[1]

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Abstract

We have shown previously that the AKT2 pathway is essential for cell survival and important in malignant transformation. In this study, we demonstrate elevated kinase levels of AKT2 and phosphatidylinositol-3-OH kinase (PI3K) in 32 of 80 primary breast carcinomas. The majority of the cases with the activation are estrogen receptor α (ERα) positive, which prompted us to examine whether AKT2 regulates ERα activity. We found that constitutively activated AKT2 or AKT2 activated by epidermal growth factor or insulin-like growth factor-1 promotes the transcriptional activity of ERα. This effect occurred in the absence or presence of estrogen. Activated AKT2 phosphorylates ERα *in vitro* and *in vivo*, but it does not phosphorylate a mutant ERα in which ser-167 was replaced by Ala. The PI3K inhibitor, wortmannin, abolishes both the phosphorylation and transcriptional activity of ERα induced by AKT2. However, AKT2-induced ERα activity was not inhibited by tamoxifen but was completely abolished by ICI 164,384, implicating that AKT2-activated ERα contributes to tamoxifen resistance. Moreover, we found that ERα binds to the p85α regulatory subunit of PI3K in the absence or presence of estradiol in epithelial cells and subsequently activates PI3K/AKT2, suggesting ERα regulation of PI3K/AKT2 through a nontranscriptional and ligand-independent mechanism. These data indicate that regulation between the ERα and PI3K/AKT2 pathway (ERα-PI3K/AKT2-ERα) may play an important role in pathogenesis of human breast cancer and could contribute to ligand-independent breast cancer cell growth.

Introduction

Breast cancer development and tumor growth are strongly associated with estrogens. The binding of an estrogen molecule to the ERα[3] induces a cascade of events, including the release of accessory proteins (*e.g.*, the heat-shock proteins), increased nuclear retention, DNA binding, and the transcription of estrogen-responsive genes, such as cyclin D1, c-myc, cathepsin D, and transforming growth factor-α that are known to stimulate mammary cell proliferation (1). ERα is a member of a superfamily of nuclear receptors that act as transcription factors when bound to specific lipophilic hormones. In common with other steroid hormone receptors, the ERα has a NH_2-terminal domain with a hormone-independent transcriptional activation function (AF-1), a central DNA-binding domain, and a COOH-terminal ligand-binding domain with a hormone-dependent transcriptional activation function (AF-2; Refs. 2, 3). In addition to its ligand, estradiol, the ERα is also activated by several nonsteroidal growth factors including EGF and IGF1 through their cell membrane receptors and cytoplasmic signaling pathways such as MAPK signal transduction pathway (3, 4). Because of the role of ERα in promoting the growth and progression of breast cancers, considerable efforts are devoted to development of reagents to functionally inactivate ERα, so as to inhibit ERα-mediated gene expression and cell proliferation. Antiestrogens such as tamoxifen and ICI 164,384 antagonize the effects of estrogens by competing with estrogen for binding to ERα. Tamoxifen or its derivative 4-hydroxytamoxifen inhibits transcriptional activation by AF-2 but not AF-1 (5). ICI 164,384, on other hand, is a complete antagonist that blocks transcriptional activation by both AF-1 and AF-2 (6). However, approximately one-third of ERα-positive breast cancers fail to respond to antiestrogen treatment, which is thought to result from growth factor-induced ERα activity through activation of protein kinases resulting in phosphorylation of ERα (7).

It has been well documented that phosphorylation of ERα is essential for the activation of ERα after stimulation with its ligand and nonsteroidal growth factors (EGF and IGF1). The phosphorylation of ERα is observed at both serine and tyrosine residues. The serine residues are the predominant modified amino acids present in ERα, and four of these (Ser-104, Ser-106, Ser-118, and Ser-167) are clustered in the NH_2 terminus within the AF-1 region. Phosphorylation of ERα at Ser-118 is mediated by the Ras/MAPK pathway; therefore, activation of the MAPK pathway enables ligand-independent transactivation of ERα (4). There is evidence showing that Ser-167 is phosphorylated by several protein kinases, including casein kinase II and $pp90^{rsk1}$, which is important for DNA binding and transcriptional activation (8, 9). Phosphorylation of ERα on tyrosine 537, which is required for ERα dimerization and transactivation, by Src family tyrosine kinases *in vitro* has also been demonstrated. Moreover, protein kinase A has been shown to phosphorylate ERα at Ser-236 and regulate its dimerization (10).

In addition, recent studies (11) demonstrated that plasma membrane ERα plays a crucial role in transducing cellular signals. It has been convincingly shown that ERα activates G-protein-coupled receptor leading to the modulation of downstream pathways that have discrete cellular actions including membrane K^+ and Ca^{2+} channel activation and induction of protein kinase C and protein kinase A kinase activity (11). A recent study (12) demonstrated that estrogen activates p38 MAPK, resulting in the activation MAPK-protein kinase-2 and subsequent phosphorylation of heat shock protein 27. ERα has been also shown to interact with IGF1R and induce IGF1R and extracellular signal-regulated kinase activation (13).

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[3] The abbreviations used are: ERα, estrogen receptor α; PI3K, phosphatidylinositol-3-OH kinase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; EGF, epidermal growth factor; IGF1, insulin-like growth factor-1; GST, glutathione *S*-transferase; HEK, human embryonic kidney.

Akt, also called protein kinase B, has been identified as a direct target of PI3K (14). All of the three members, Akt/AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ, of this family are activated by growth factors in a PI3K-dependent manner (14–16). Numerous studies (17) showed that the Akt pathway is critical for cell survival by phosphorylation of a number of downstream proteins including BAD, caspase-9, Forkhead transcription factors, IKKα, Raf, and p21-activated protein kinase. Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies such as ovarian and pancreatic cancers (18–20). In this study, we demonstrate frequent activation of AKT2 and PI3K in human breast cancer. AKT2 phosphorylates ERα at Ser-167 and activates ERα-mediated transcription in a PI3K-dependent manner. ERα binds to the p85α subunit of PI3K in epithelial cells and activates the PI3K/AKT2 pathway in an estrogen-independent manner.

Materials and Methods

Tumor Specimens, Cell Lines, and Transfection. All of the 80 primary human breast cancer specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center, and each sample contained at least 70% tumor cells, as was confirmed by microscopic examination. The tissues were snap-frozen and stored at −70°C. ERα-negative epithelial HEK293 and COS7 cells and ERα-positive MCF7 and BG-1 cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS. The cells were seeded in 60-mm Petri dishes at a density of 8×10^5 cells/dish. Transfections were performed by calcium phosphate DNA precipitation or Lipofectamine Plus (Life Technologies, Inc.).

Immunoprecipitation and Western Blotting Analysis. The cells and frozen tumor tissues were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (volume for volume) glycerol, 1% NP40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM $NaPP_i$, 1 mM sodium vanadate, and 25 mM β-glycerol phosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4°C before immunoprecipitation or Western blotting. The protein concentration in each tissue lysate was measured, and an equal amount of protein was analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 min. After removal of the beads by centrifugation, lysates were incubated with anti-AKT2 (Upstate Biotechnology) antibody in the presence of 30 μl of protein A-protein G (2:1) agarose beads for 2 h at 4°C. The beads were washed once with 50 mM Tris-HCl (pH 7.5)-0.5 M LiCl-0.5% Triton X-10, twice with PBS, and once with 10 mM Tris-HCl (pH 7.5)-10 mM $MgCl_2$-10 mM $MnCl_2$-1 mM DTT, all containing 20 mM β-glycerol phosphate and 0.1 mM sodium vanadate. Immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein phosphorylation and expression were determined by probing Western blots of immunoprecipitates with anti-phospho-Akt-Ser473 (Cell Signaling) or anti-AKT2 antibody. Detection of antigen-bound antibody was carried out with the enhanced chemiluminescence Western Blotting Analysis System (Amersham).

***In Vitro* Protein Kinase Assay.** Akt kinase assay was performed as described previously (15). Briefly, the reaction was carried out in the presence of 10 μCi of [γ-^{32}P]ATP (NEN) and 3 μM cold ATP in 30 μl of buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM DTT using histone H2B as substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein-loading buffer, and the mixture was separated in SDS-PAGE gels. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Phosphorimager (Molecular Dynamics).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven after dewaxing and rehydration. The Vectastain ABC Kit for sheep IgG (Vector Laboratories) was used to immunostain the tissue sections with phospho-S473 Akt antibody (catalogue number 06-801-MN; Upstate Biotechnology). Endogenous peroxidase and biotin were blocked, and sections were incubated 1 h at room temperature with a 1:250 dilution of antibody to phospho-S473 Akt. The remainder of the staining procedure was performed according to the manufacturer's instructions using diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining. Primary antibody was replaced with an equal concentration of nonimmune sheep IgG on negative control sections.

PI3K Assay. PI3K was immunoprecipitated from the tumor tissue lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), and finally with 10 mM Tris, 100 mM NaCl, 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP, 20 μCi [γ-^{32}P]ATP, and 10 μg of L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2; BIOMOL) for 20 min at 25°C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$/methanol. Phosphorylated products were separated by TLC as described previously (21). The conversion of PI-4,5-P_2 to PI-3,4,5-P_3 was determined by autoradiography and quantitated by using a Phosphorimager. Average readings of the kinase activity 3-fold higher than that in normal ovarian tissue was considered as elevated PI3K activity.

Expression Constructs and GST Fusion Protein. HA epitope-tagged constitutively active, wild-type, and dominant-negative AKT2 were prepared as described previously (21). The p110α and p85α of PI3K expression constructs were gifts from Dr. Julian Downward (Imperial Cancer Research Fund, London, United Kingdom). The mammalian expression construct of ERα-S167A was kindly provided by Dr. Benita S. Katzenellenbogen (University of Illinois, Urbana, IL). The GST-ERα and GST-ERα-S167A were created by PCR and inserted into pcDNA3 and pEGX-4T (Pharmacia) vectors, respectively. GST-ERα fusion proteins were purified as described previously (21).

***In Vivo* [^{32}P]P_i Cell Labeling.** Transfected COS7 and nontransfected MCF7 cells were labeled with [^{32}P]P_i (0.5 mCi/ml) in MEM without phosphate, serum, and phenol red for 4 h and lysed. ERα was immunoprecipitated with monoclonal anti-ERα or anti-myc antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated ERα was detected by autoradiography and quantitated by using Molecular Dynamics Phosphorimager with ImageQuant software.

Reporter Assay. HEK293 and MCF7 cells (8×10^5) were seeded in a 60-mm plate. The cells were cotransfected with the luciferase reporter plasmid (2ERE-MpG12), wild-type, constitutively active, or dominant-negative AKT2 and ERα, as well as pCMV-βgal plasmid as an internal control. The amount of DNA in each transfection was kept constant by the addition of empty pcDNA3 vector. Luciferase and β-galactosidase activities were determined 48 h after transfection according to the manufacturer's procedure (Promega). Luciferase activity was corrected for transfection efficiency by using the control β-galactosidase activity. All of the experiments were performed in triplicate from independent cell cultures.

Results and Discussion

Frequent Activation of AKT2 in Breast Carcinoma. We have demonstrated previously (15, 20) that AKT2, like AKT1, is activated by a number of mitogenic growth factors in a PI3K-dependent manner and that AKT2 kinase activity is frequently elevated in human ovarian tumors. To examine whether AKT2 is activated in human primary breast cancer, we performed *in vitro* kinase assays in 80 human breast carcinoma specimens, including 58 ductal infiltrating adenocarcinomas, 16 lobular carcinomas, and six mixed tumors. Lysates from tumor specimens were incubated with anti-AKT2 antibody, which specifically reacts with AKT2 (20). The immunoprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate. The results revealed an elevated level of AKT2 kinase in 32 of specimens (40%), including 29 cases with ductal infiltrating carcinoma, two lobular, and one mixed tumor (Fig. 1*A*). To further demonstrate AKT2 activation in breast cancer, we performed Western blot analyses of tumor lysates with phospho-Ser-473 antibody, a phosphorylation site that is critical for activation of three isoforms of Akt (17). To avoid the cross-reaction, the tumor lysates were incubated with anti-AKT2 antibody. The AKT2 immunoprecipitates were separated by SDS-PAGE and probed with phospho-Ser473 antibody. Phosphorylated AKT2 was detected only in breast tumors with elevated AKT2 kinase

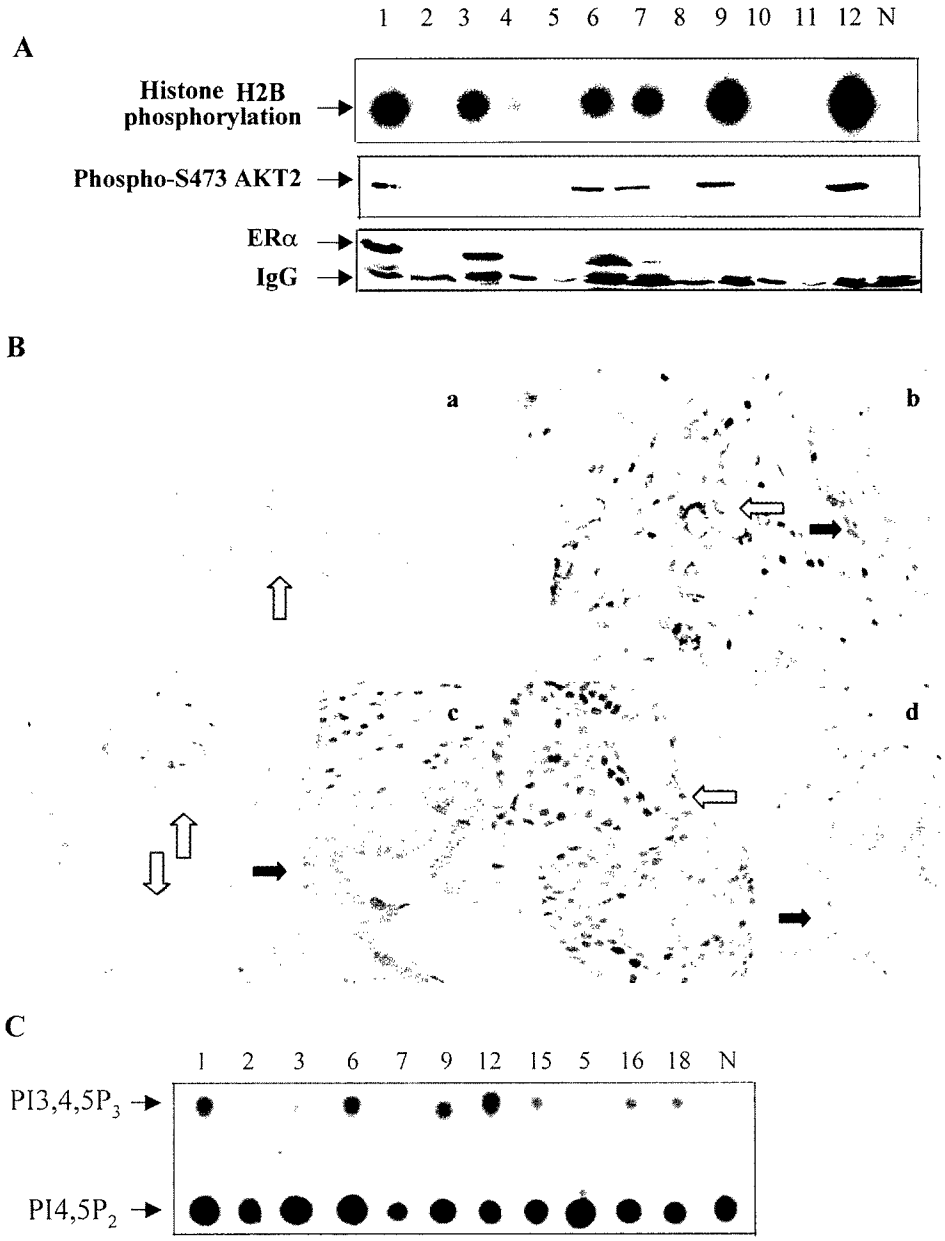

Fig. 1. Activation of AKT2 in human primary breast cancers. *A* (*top panel*), *in vitro* kinase assays of immunoprecipitated AKT2 from representative frozen breast tumor specimens. Normal mammary tissue (*N*) was used as a control. *Bottom panels*, Western blot analyses of AKT2 and ERα immunoprecipitates with anti-phospho-Ser473 Akt and anti-ERα antibodies, respectively. *B*, immunochemical staining of the paraffin sections prepared from primary breast adenocarcinomas with anti-phospho-S473 Akt (*a–c*) and anti-ERα (*d*) antibodies. Strong staining with both antibodies was observed in tumor cells (*white arrows*), whereas weak immunoreaction was detected in stromal tissue and adjacent ductal epithelium (*black arrows*). Photomicrographs *c* and *d* are the same specimen but different sections. *C*, *In vitro* PI3K assay of anti-p85 immunoprecipitates from 11 tumor and one normal specimen. The specimen numbers correspond to the same tumors shown in *A*.

activity (Fig. 1*A*). Because stromal tissues account for approximately 20–30% of the tumor specimens used in this study, we examined whether the activation of AKT2 is derived from the tumor cells or the stromal tissues by immunostaining paraffin sections with a phospho-Ser473 Akt antibody. Positive staining of tumor cells was detected in all of the 32 cases with AKT2 activation, whereas no staining was observed in normal ductal epithelial cells (Fig. 1*B*). These data suggest that activation of AKT2 is a common occurrence in human breast cancer.

Because AKT2 is a downstream target of PI3K, which is activated in colon and ovarian carcinoma (20, 22, 23), we next examined the PI3K activity in breast tumors by *in vitro* PI3K assay. Because of the fact that all of the tumors with elevated PI3K activity result in activation of Akt (20, 22–24), immunoprecipitation with a pan-p85 antibody was performed in 58 breast tumor specimens, including 32 with AKT2 activation and, as control, 26 without AKT2 activation. The ability to convert PI-4,5-P_2 to PI-3,4,5-P_3 was determined. Elevated PI3K activity was detected in all of the 32 specimens that exhibited AKT2 activation. No PI3K activation was observed in 26 specimens without AKT2 activation (Fig. 1*C*), indicating that activation of AKT2 in breast cancer predominantly results from PI3K activation. Moreover, Western blot and immunohistochemistry analyses with anti-ERα antibody revealed that 88% of the cases (28 of 32) with PI3K/AKT2 activation showed strong ERα positive (Fig. 1, *A* and *B*), whereas only 54% of the cases (14 of 26) without PI3K/AKT2 activation exhibited positive ERα, suggesting that activated PI3K/AKT2 could be involved in the regulation of ERα activity in breast cancer cells. In addition, the majority of cases with AKT2 activation are late stage (23 of 32 at stages III and IV) and poorly differentiated tumors (19 of 32), indicating that PI3K/AKT2 activation in breast cancer may be associated with tumor progression rather than initiation.

AKT2 Activates ERα-mediated Transcription in a Ligand-independent Manner. Previous studies (1, 25) have shown that MAPK is activated in breast cancer and contributes to estrogen-independent breast tumor cell growth by direct phosphorylation of ERα. Moreover, several other signal molecules, including protein kinase A, casein kinase II, $pp90^{rsk1}$, and MEKK1/p38, have been

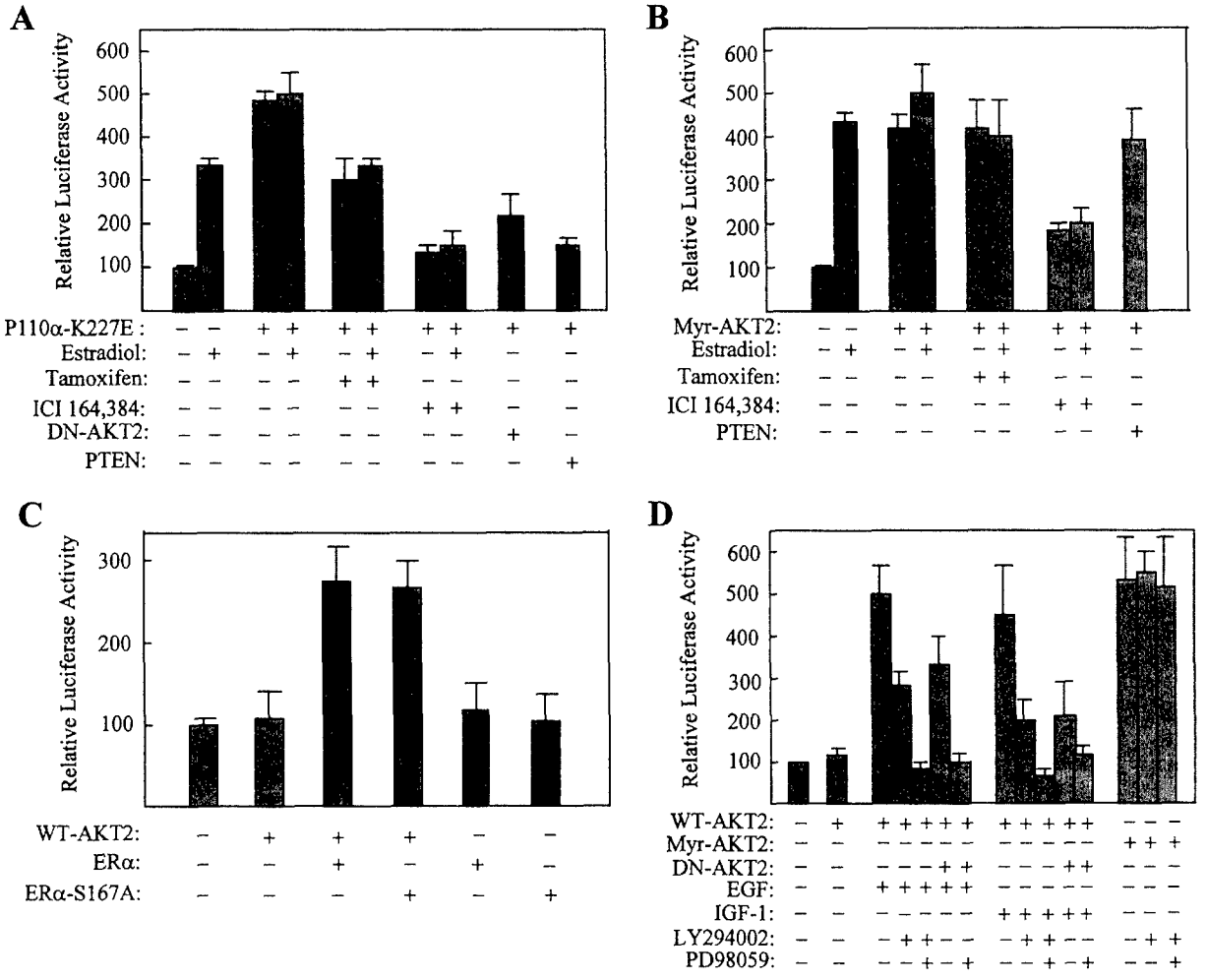

Fig. 2. AKT2 and PI3K activate ERα transcriptional activity. *A–D*, reporter assays: MCF-7 cells were transfected with ERE2-TK-LUC reporter, β-galactosidase, and indicated expression constructs. After 36 h of transfection, the cells were serum-starved overnight and treated with indicated agents. Luciferase activity was normalized to β-galactosidase activity.

shown to activate ERα-mediated transcription, possibly resulting in hormone-independent tumor cell growth (1, 8–10, 26). Because AKT2 and PI3K are frequently activated in breast cancer and the majority of cases with AKT2 activation are ERα positive, we investigated whether AKT2 and PI3K regulate ERα-mediated transcription. ERα-positive MCF7 breast cancer cells were transiently transfected with a reporter construct containing a luciferase gene regulated by two estrogen response elements (ERE2-TK-LUC) and a plasmid expressing β-galactosidase that allows the luciferase data to be normalized for transfection efficiency. In addition, the cells were transfected with expression constructs for constitutively activated p110α (p110α-K227E) subunit of PI3K, wild-type, constitutively activated, and dominant-negative AKT2 or vector alone. As shown in Fig. 2, p110α-K227E or myr-AKT2 increased ERE2-TK-LUC activity 3–4-fold in the absence of estradiol. Constitutively activated p110α-induced reporter activity was attenuated by dominant-negative mutant AKT2 (Fig. 2*A*). Tamoxifen (4-hydroxytamoxifen), an antiestrogen reagent that inhibits transcriptional activation by AF2 but not through AF1 (5), abolished estradiol-enhanced transcription but had no effects on p110α-K227E and myr-AKT2-stimulated ERα activity (Fig. 2, *A* and *B*), suggesting that PI3K/AKT2-increased ERα transcriptional activity is regulated by phosphorylation of ERα within the AF1 region and could be involved in tamoxifen resistance.

ICI 164,384, which causes rapid degradation of ERα (6, 27), completely blocked PI3K- and AKT2-induced reporter activity. PTEN, a tumor suppressor encoding a lipid phosphatase that negatively regulates PI3K, inhibited constitutively active p110-induced ERα-mediated transcription but had no effect on constitutively activated AKT2-stimulated ERα activity (Fig. 2*B*).

Moreover, we have observed that exogenous expression of ERα in ERα-positive MCF7 cells increased wild-type AKT2-induced ERE2-TK-LUC activity 2–3-fold as compared with cells transfected with wild-type AKT2 alone (Fig. 2*C*), implying that ERα might activate AKT2 kinase and subsequently enhance its own transcriptional activity (see below). Taken collectively, these data indicate that PI3K/AKT2-activated ERα-mediated transcription is estrogen-independent and that the frequently elevated level of PI3K/AKT2 kinase in primary breast cancer could relate the refractoriness of hormone therapy.

AKT2 Mediates Growth Factor-induced ERα Transcriptional Activity. A very recent study (28) showed that Akt1 mediates the estrogenic functions of EGF and IGF1. Next, we examined the possible role of AKT2 in growth factor-induced ERα activation. ERα-positive MCF7 cells were transfected with ERE2-TK-LUC and dominant-negative, wild-type, or constitutively activated AKT2 or vector alone and were treated with or without either 100 ng/ml EGF or 50 ng/ml IGF1 (Fig. 2*D*). Treatment with the growth factors resulted in an approximately 4.5-fold increase in ERα-mediated transcriptional activity. The EGF- and IGF1-induced reporter activity was partially abrogated by dominant-negative AKT2 or PI3K inhibitor LY294002 and completely blocked by the combination of PI3K and MAPK inhibitors (LY294002 and PD98059). However, the combined inhibitors had no effect on constitutively activated AKT2-induced reporter

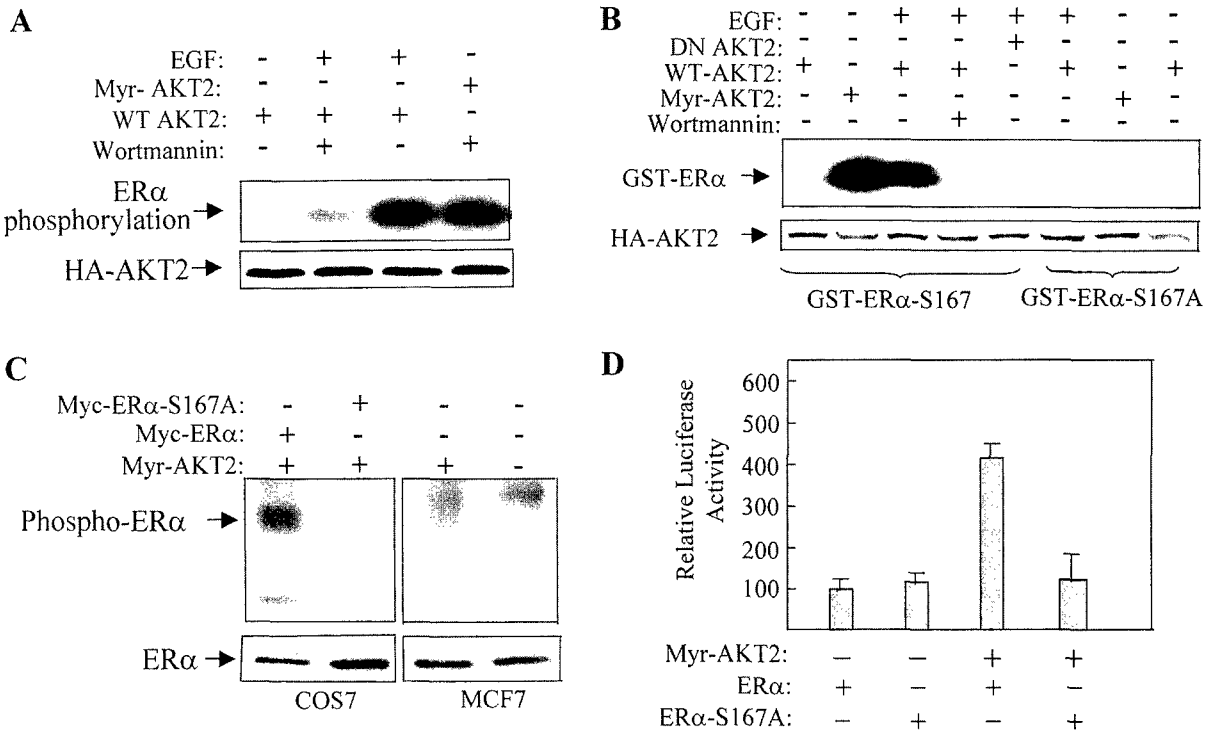

Fig. 3. AKT2 phosphorylates ERα on serine-167 *in vitro* and *in vivo*. *In vitro* AKT2 kinase assay of the immunoprecipitates from HEK293 cells transfected with indicated expression constructs. Full length of human recombinant ERα (*A*), GST-ERα-S167, and GST-ERα-S167A (*B*) were used as substrates. *C*, COS7 and MCF7 cells were transfected with indicated plasmids and incubated with [^{32}P]P_i for 4 h. Immunoprecipitates were prepared with anti-myc (*left*) or anti-ERα (*right*) antibody and separated by SDS-PAGE. After transfer, the membrane was exposed to a film (*top*) and detected with anti-ERα antibody (*bottom*). *D*, AKT2 phosphorylation of serine-167 is essential for AKT2-induced ERα transcriptional activity. Luciferase reporter assay of HEK293 cells transfected with ERE2-TK-LUC, wild-type ERα, or ERα-S167A, β-galactosidase, and myr-AKT2.

activity. These results suggest that the "steroid-independent activation" of ERα by growth factors is mediated by the PI3K/AKT2 pathway, in addition to MAPK, PKA, casein kinase II, and pp90^{rsk1}.

AKT2 Phosphorylated Serine-167 of ERα *in Vitro* and *in Vivo*. Phosphorylation of ERα has been shown to be an important mechanism by which ERα activity is regulated. ERα is hyperphosphorylated on multiple sites in response to hormone binding and growth factor stimulation (1–4). Transcriptional activation by growth factors has been shown to require AF-1 but not AF-2 (1, 8–10). There is evidence to suggest that EGF and IGF-1 induce MAPK and pp90^{rsk1}/casine kinase II activity leading to phosphorylation of serine-118 and serine-167, respectively, in AF-1 region (4, 5, 8–10). To examine whether AKT2 phosphorylates ERα *in vitro*, HEK293 cells were transfected with HA-tagged wild-type and constitutively activated AKT2, and immunoprecipitation was prepared with anti-HA antibody. *In vitro* AKT2 kinase assays, using full length of human recombinant ERα as substrate, revealed that constitutively activated AKT2 and EGF-induced AKT2 strongly phosphorylated hERα. The ERα phosphorylation that was induced by EGF-stimulated AKT2 was abrogated by wortmannin (Fig. 3*A*).

To determine whether AKT2 phosphorylates ERα *in vivo*, MCF7 cells were transfected with constitutively activated AKT2 or pcDNA3 vector alone and labeled with [^{32}P]P_i. The cell lysates were incubated with anti-ERα antibody, and the immunoprecipitates were separated on SDS-PAGE. ERα was highly phosphorylated in constitutively activated AKT2-transfected cells but in the cells transfected with vector alone (Fig. 3*C*). These data indicate that AKT2 phosphorylated ERα both *in vitro* and *in vivo*.

Martin *et al.* (28) recently demonstrated that EGF- and IGF1-induced Akt1 potentiates the AF-1 function of ERα, possibly through the phosphorylation of serine residues. There are four serine residues (Ser-104, Ser-106, Ser-118, and Ser-167) in the AF-1 region of the receptor that are predominantly phosphorylated in response to estrogen and growth factor stimulation (1–4). We examined the ERα protein sequence and found that serine-167 (^{162}**RERLAS**^{167}) is a putative AKT2 phosphorylation site. Constructs expressing GST-fused wild-type and mutant (S167A) AF-1 region were created. *In vitro* kinase assays revealed that myr-AKT2 and EGF-stimulated AKT2 strongly phosphorylated GST-ERα-S167 but not GST-ERα-S167A mutant (Fig. 3*B*). The EGF-induced AKT2 phosphorylation of ERα is blocked by wortmannin. To examine whether AKT2 phosphorylates serine-167 *in vivo*, COS7 cells were transfected with myc-tagged wild-type and mutant (S167A) human ERα expression constructs together with constitutively activated AKT2. After 36 h of transfection, the cells were incubated with [^{32}P]P_i and immunoprecipitated with anti-myc antibody. As demonstrated in Fig. 3*C*, constitutively active AKT2 phosphorylated wild-type ERα but not the ERα-S167A mutant *in vivo*, suggesting that serine-167 of ERα is a physiological substrate for AKT2.

Previous studies (29) showed that serine-167 is important for ERα transcriptional activity. To further examine whether AKT2-activated ERα transcriptional activity depends upon phosphorylation of serine-167, reporter assays were carried out in HEK293 cells transfected with ERE2-TK-LUC, constitutively activated AKT2, and ERα-S167A or wild-type ERα. Fig. 3*D* shows that ERα-S167A had no ability to mediate constitutively activated AKT2-induced ERE2-TK-LUC reporter activity, indicating that AKT2 regulates ERα-mediated transcription through phosphorylation of serine-167.

ERα Binds To and Activates PI3K/AKT2 in Epithelial Cells via a Ligand-independent Mechanism. Recent studies (30, 31) demonstrated that ERα binds to the p85α regulatory subunit of PI3K after estradiol treatment, leading to the activation of PI3K/Akt and endo-

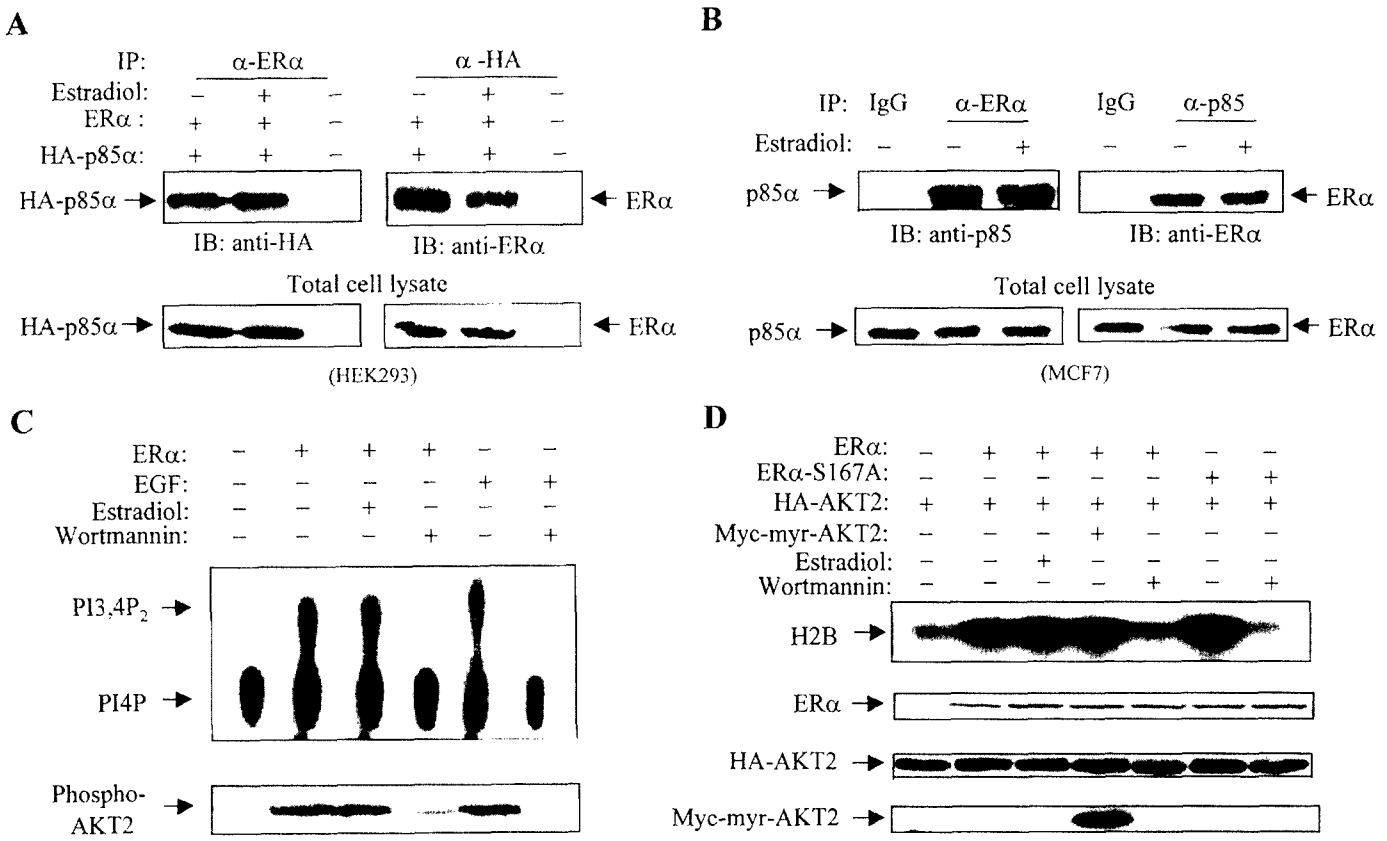

Fig. 4. ERα interacts with p85α and activates the PI3K/AKT2 pathway in human epithelial cells. Coimmunoprecipitation of ERα and p85α in (*A*) HEK293 cells cotransfected with HA-p85α/ERα and in (*B*) nontransfected MCF7 cells. *Top*, coimmunoprecipitation; *bottom*, Western blot of total cell lysates. *C*, *in vitro* PI3K assay (*top*) of HEK293 cells transfected and treated with indicated plasmid and agents. *Bottom*, Western blotting analysis of AKT2 immunoprecipitates with phospho-S473 Akt antibody. *D*, *in vitro* kinase assay (*top*) of HA-AKT2 immunoprecipitates prepared from HEK293 cells transfected with indicated expression constructs, using histone H2B as a substrate. *Panels 2–4*, Western blots of transfected HEK293 cell lysates detected with anti-ERα, anti-HA, or anti-myc antibody.

thelial nitric oxide synthase in endothelial cells. In the absence of estradiol, ERα failed to bind and activate PI3K, indicating that ERα-associated PI3K in endothelial cells is estrogen-dependent (30). Next, we examined whether ERα binds to and activates PI3K/AKT2 in epithelial cells. ERα/HA-p85α-transfected HEK293 and nontransfected ERα-positive MCF7 cells were immunoprecipitated with anti-ERα and detected with anti-HA or anti-p85α antibody or *vice versa*. As shown in Fig. 4*A* and *B*, ERα constitutively associated with p85α, and this interaction was unaffected by estradiol treatment. In addition, *in vitro* PI3K assays revealed that expression of ERα in HEK 293 cells significantly induced PI3K activity in the absence or presence of estradiol (Fig. 4*C*). These data suggest that ERα binding to and activating PI3K is ligand-independent in epithelial cells.

Next, we examined whether ERα activates AKT2 and whether this activation is dependent on AKT2 phosphorylation. ERα-negative HEK293 cells were transfected with ERα or ERα-S167A, together with HA-AKT2. *In vitro* AKT2 kinase assays revealed that ERα significantly activates AKT2 in the absence of estradiol. Additional estradiol treatment did not further enhance ERα-induced AKT2 activation. The PI3K inhibitor, wortmannin, completely abolished the activation. Interestingly, ERα-S167A activated AKT2 at a similar level to that of wild-type ERα. Coexpression of myc-tagged constitutively active AKT2 (Myc-myr-AKT2) and ERα had the same effect on wild-type AKT2 activation as that of expression of ERα alone (Fig. 4*D*). These results indicate that activation of AKT2 by ERα is through PI3K and independent of ERα phosphorylation by PI3K/AKT2.

In summary, we demonstrate in this study that AKT2 and PI3K are frequently activated in primary human breast carcinoma. The PI3K/AKT2 pathway regulates ERα transcriptional activity by phosphorylation of serine-167 *in vitro* and *in vivo*, and ERα activates PI3K/AKT2 kinase by binding to p85α in a ligand-independent manner in epithelial cells. This study suggests that the PI3K/AKT2 pathway may play a pivotal role in estrogen-independent breast cancer cell growth and tamoxifen-resistance; therefore, it could represent an important therapeutic target in human breast cancer.

Acknowledgments

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Inhibition of JNK by Cellular Stress- and TNFα-induced AKT2 through Activation of the NFκB Pathway in Human Epithelial Cells

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[1]The abbreviations used are: PDGF, platelet derived growth factor; TNFα, tumor necrosis factor α; EGF, epidermal growth factor; IGF1, insulin-like growth factor-1; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; HA, hemagglutinin; JNK, c-Jun N-terminal kinase; IKK, IκB kinase; NIK, NFκB inducing kinase; PBS, phosphate-buffered saline; UV, ultraviolet; GST, glutathione *S*-transferase; MAP, mitogen-activated protein

Previous studies have demonstrated that AKT1 and AKT3 are activated by heat shock and oxidative stress via both phosphatidylinositol 3-kinase (PI3K)-dependent and -independent pathways. However, the activation and role of AKT2 in the stress response have not been fully elucidated. In this study, we show that AKT2 in epithelial cells is activated by UV-C irradiation, heat shock, and hyperosmolarity, as well as by tumor necrosis factor α (TNFα) through PI3K-dependent pathway. The activation of AKT2 inhibits UV- and TNFα–induced c-Jun N-terminal kinase (JNK) and p38 activities that have been shown to be required for stress- and TNFα-induced programmed cell death. Moreover, AKT2 interacts with and phosphorylates IKKα. The phosphorylation of IKKα and activation of NFκB mediates AKT2 inhibition of JNK but not p38. Furthermore, PI3K inhibitor or dominant negative AKT2 significantly enhances UV- and TNFα-induced apoptosis, whereas expression of constitutively active AKT2 inhibits programmed cell death in response to UV and TNFα stimulation with accompanying decreased JNK and p38 activity. These results indicate that activated AKT2 protects epithelial cells from stress- and TNFα-induced apoptosis by inhibition of stress kinases and provide the first evidence that Akt inhibits stress kinase JNK through activation of NFκB pathway.

Exposure of cells to environmental stress results in the activation of several signal transduction pathways including the MEKK4/MKK7/JNK, MKK3/MKK6/p38, and IKK/IκB/NFκB cascades. Stress-induced clustering and internalization of cell surface receptors, such as those for platelet-derived factor (PDGF), tumor necrosis factor α (TNFα), epidermal growth factor (EGF), and insulin-like growth factor 1 (IGF1), mediate stress-kinase activation (1-3). Recent studies suggest that nearly all stress stimuli activate phosphotidylinositol 3-kinase (PI3K) (1), and of the downstream targets of PI3K, Akt is thought to play an essential role in the cellular response to stress.

Akt, also termed protein kinase B (PKB) or RAC kinase, represents a family of PI3K regulated serine/threonine kinases (4, 5). Three different isoforms of Akt have been identified: AKT1/PKBα (AKT1), AKT2/PKBβ (AKT2), and AKT3/PKBγ (AKT3), all of which are activated by growth factors in a PI3K dependent manner (4-9). Full activation of the Akts requires their phosphorylation at Thr^{308} (AKT1), Thr^{309} (AKT2), or Thr^{305} (AKT3) in the activation loop and Ser^{473} (AKT1), Ser^{474} (AKT2), or Ser^{472} (AKT3) in the C-terminal activation domain (9). AKT1, the most studied isoform which was originally designated as Akt, suppresses apoptosis induced by a variety of stimuli, including growth factor withdrawal and loss of cell adhesion. Possible mechanisms by which AKT1 promotes cell survival include phosphorylation and inactivation of the proapoptotic proteins BAD and caspase-9 (10, 11). AKT1 also phosphorylates and inactivates the Forkhead transcription factors, an event that results in the reduced expression of the cell cycle inhibitor, $p27^{Kip1}$, and the Fas ligand (12-14). Via phosphorylation of IκB kinase (IKK), AKT1 also activates NFκB, a transcription factor that has been implicated in cell survival (15, 16).

Two separate studies demonstrated that AKT1 is activated when NIH 3T3 fibroblasts are stressed in a variety of ways (17, 18). Based on data showing that PI3K inhibitors do not prevent AKT1 activation by stress, these studies concluded that stress-induced AKT1 activation was PI3K-independent. Other studies, however, found that PI3K activity was required for AKT1 activation by heat shock or oxidative stress in Swiss 3T3 cells (19, 20). It has been suggested that certain cellular stresses activate AKT1 and AKT3 but not AKT2 (19), a finding that is consistent with the different functions of the AKTs as revealed by studies of mice lacking Akt1 or Akt2 (21-23). Nevertheless, activation of AKT2 by stress and the role of AKT2 in the stress response have yet to be fully explored. The data presented here show that AKT2 is significantly activated by stress stimuli (e.g., UV irradiation, heat shock, and hyperosmolarity) and by TNFα in human epithelial cells but not in fibroblasts. Stress-induced AKT2 activation in epithelial cells is completely blocked by inhibitors of PI3K. When activated by stress, AKT2 inhibits JNK and p38 activities that are required for stress-induced apoptosis. In addition, AKT2 binds to and phosphorylates IKKα and consequently activates NFκB resulting in inhibition of programmed cell death in response to stress stimuli. Moreover, AKT2-induced NFκB activation is required for the inhibition of JNK, but not p38, activity.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection, and Stimulation – The human epithelial cancer cell lines, A2780, OVCAR3 and human embryonic kidney (HEK) 293 cells were cultured at 37^0C and 5% CO_2 in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The cells were seeded in 60 mm Petri dishes at a density of 0.5×10^6 cells per dish. Following incubation

overnight, the cells were transfected with 2 μg of DNA per dish using LipofectAMINE Plus (Invitrogen Life Technologies). After 36 h of the transfection, the cells were serum-starved overnight and stimulated with UV-C irradiation, heat (45^0C), 0.4 M NaCl, or 20 to 50 ng/ml TNFα.

Expression Constructs – The cytomegalovirus (CMV)-based expression constructs encoding wild type HA-AKT2, constituitively active HA-Myr-AKT2, and dominant negative HA-E299K-AKT2 have been described (24). The HA-JNK1 construct was kindly provided by Michael Karin (School of Medicine, University of California at San Diego). GST-c-Jun (1-79) and pCMV-Flag-p38 were gifts from Roger J. Davis (School of Medicine, University of Massachusetts). The constructs used in the study of the NFκB pathway were prepared as previously described (25).

Immunoprecipitation and Immunoblotting – Cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (vol/vol) glycerol, 1% NP-40, 2 mM phenylmethylsufonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM NaPPi, 1 mM sodium vanadate, and 25 mM β-glycerolphosphate. Lysates were centrifuged at 12,000 g for 15 min at 4^0C prior to immunoprecipitation or Western blotting. Aliquots of the cell lysates were analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4^0C for 20 min. Following the removal of the beads by centrifugation, lysates were incubated with anti-HA monoclonal antibody 12CA5 (Roche Diagnostics), anti-flag antibody (Sigma), or anti-AKT2 antibody (Santa Cruz Biotechnology) in the presence of 30 μl of protein A-protein G

(2:1) agarose beads for 2 h at 4^0C. The beads were washed once with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.5% Triton X-10, twice with phosphate buffered saline (PBS), and once with buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM DTT, all supplemented with 20 mM β-glycerolphosphate and 0.1 mM sodium vanadate. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates or total cell lysates with the antibodies described above or with the appropriate antibodies as noted in figure legends. Detection of antigen-bound antibody was carried out with the ECL Western Blotting Analysis System (Amersham).

In vitro Protein Kinase Assay – Protein kinase assays were performed as previously described (26, 27). Briefly, reactions were carried out in the presence of 10 μCi of [γ-^{32}P] ATP (Perkin Elmer Life Sciences) and 3 μM cold ATP in 30 μl buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. Histone H2B was used as exogenous substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein loading buffer and proteins were separated on SDS-PAGE gels. Each experiment was repeated three times and the relative amounts of incorporated radioactivity were determined by autoradiography and quantitiated with a Phosphoimager (Molecular Dynamics).

PI3K Assay – PI3K was immunoprecipitated from the cell lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl/0.1 M Tris (pH 7.4), and finally with 10 mM Tris/100 mM NaCl/1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads in

reaction buffer (10 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 50 μM ATP) containing 20 μCi [γ-^{32}P] ATP and 10 μg L-α-phosphatidylinositol 4, 5-bis phosphate (Biomol) for 20 min at 25^{0}C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl $CHCl_3$/MeOH and phosphorylated products were separated by thin-layer chromatography as previously described (24). The conversion of PI 4,5-P_2 to PI 3,4,5-P_3 was detected by autoradiography and quantitiated with a Phosphoimager.

NFκB Transcriptional Activation Analysis – HEK293 cells were seeded in 60-mm dishes and transfected with 1.5 μg NFκB reporter plasmid (pElam-luc), 0.8 μg pSV2-β-gal and different forms (wild-type, constitutively active, or dominant-negative) of HA-AKT2 or vector alone. The total amount of DNA transfected was increased to 6 μg with empty vector DNA. Following serum starvation overnight, the cells were treated with UV (40J/m^2) or TNFα (20 ng/ml), and lysed with 400 μl/dish of Reporter Lysis buffer (Tropix). The cell lysates were cleared by centrifugation for 2 min at 4^{0}C. Luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix), respectively. Each experiment was repeated three times.

TUNEL Assay – AKT2 stably-transfected A2780 cells were seeded into 60-mm dishes and grown in Dulbecco modified Eagle medium; supplemented with 10% FCS for 24 h, pretreated with or without LY294002 for 2 h prior to exposure to UV, heat shock, NaCl, or TNFα. Apoptosis was determined by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) using an *in situ* cell death detection kit (Boehringer Mannheim,

Indianapolis, IN). The cells were trypsinized, and cytospin preparations were obtained. Cells were fixed with freshly prepared paraformaldehyde (4% in PBS, pH 7.4). Slides were rinsed with PBS, incubated in permeabilization solution, followed by TUNEL reaction mixture for 60 min at 37°C in a humidified chamber. After a rinse, the slides were incubated with converter-alkaline phosphatase solution for 30 min at 37°C and then detected with alkaline phosphatase substrate solution (Vector Laboratories, Burlingame, CA) for 10 min at 25°C. After an additional rinse, the slides were mounted and analyzed under a light microscope. These experiments were performed in triplicate.

RESULTS

AKT2 Is Activated by UV Irradiation, Heat Shock, hyperosmolarity and TNFα – Previous studies showed that stress activates AKT1 and AKT3 but not AKT2 in fibroblasts (19). It has also been shown that TNFα receptor mediates UV- and heat shock-induced stress signaling (1-3). In agreement with these studies, we found that exposure of NIH 3T3 fibroblasts to UV-C, heat, or hyperosmotic conditions did not result in AKT2 activation (data not shown). It is possible, however, that stress might activate AKT2 in epithelial cells due to the fact of frequent alterations of AKT2, but not AKT1 and AKT3, in human epithelial tumors (7, 24, 27). For this reason we examined the effects of stress on AKT2 activation in two ovarian epithelial cancer cell lines: A2780 cells, which were transiently transfected with HA-AKT2, and OVCAR3 cells, which express high levels of endogenous AKT2 (7). The cells were exposed to UV-C, heat shock (45^0C), 0.4 M NaCl, or 20 ng/ml TNFα. IGF1-stimulated cells were used as controls. As assessed by *in vitro* kinase and Western blot analyses of AKT2 immunoprecipitates, all the

stimuli substantially increased AKT2 activity in both A2780 and OVCAR3 cells (Figs. 1A and 1B). The levels of AKT2 activity induced by these agents, however, were variable. AKT2 activity induced by TNFα and UV was comparable to that stimulated by IGF-1, whereas the effect of heat shock and hyperosmolarity (NaCl) on AKT2 activity was relatively smaller (Fig. 1). Nevertheless, these findings suggest that stresses activate AKT2 in a cell type-specific manner.

Stress Simulates PI3K that Mediates AKT2 Activation – To show that stress does indeed activate PI3K in epithelial cells, A2780 or HEK293 cells were exposed to UV irradiation, heat shock, 0.4 M NaCl or TNFα, and cell lysates immunoprecipitated with antibody to pan-p85, a regulatory subunit of PI3K. Assay of PI3K activity shows that these stress conditions, as well as TNFα, activated PI3K as efficiently as did IGF-1 (Fig. 2A). As described above, stress has been shown to activate AKT1 by both PI3K-dependent and -independent pathways (17, 18). To assess the role of PI3K in the stress-induced activation of AKT2, A2780 cells transfected with HA-AKT2 were exposed to 25μM LY294002, a specific PI3K inhibitor, for 30 min prior to stress or TNFα treatments. LY294002 effectively inhibited stress- and TNFα-induced AKT2 activation (Fig. 2B). These data provide direct evidences of stress-induced activation of AKT2 through a PI3K-dependent pathway in human epithelial cells.

Stress-induced AKT2 Activation Inhibits UV- and TNFα-induced JNK and p38 Activities – Previous studies demonstrated that two groups of mitogen-activated protein (MAP) kinases, the JNK and p38, are activated by environmental stress and TNFα (28). Therefore, we examined the effects of stress-induced AKT2 activation on the JNK and p38 to determine whether stressed-

induced AKT2 activation could target these two stress kinases. A2780 cells were transfected with constitutively active AKT2 or pcDNA3 vector alone. Thirty-six hours after transfection, cells were treated with TNFα or UV, and analyzed by Western blot for JNK and p38 activation using anti-phospho-JNK and anti-phospho-p38 antibodies. Both JNK and p38 were activated by TNFα and UV irradiation. The maximal activation was observed at 10 min of the stimulation. Expression of constitutively active AKT2, however, exhibited inhibitory effects on the activation of JNK and p38 that was induced by TNFα and UV irradiation. Notably, the activation of JNK and p38 in constitutively active AKT2-transfected cells does not significantly differ from that of the cells transfected with pcDNA3 vector at 10 min of TNFα treatment. However, the phosphorylation levels of JNK and p38 in the cells expressing constitutively active AKT2 declined much greater than that of pcDNA3-transfected cells after 30 min of the stimulation (Fig. 3 and data not shown). We, therefore, conclude that the activation of AKT2 does not activate but rather inhibits TNFα- and UV-induced JNK and p38 activities.

AKT2 interacts with and phosphorylates IKKα, but not NIK, leading to IκBα degradation and NFκB activation – The capacity of both cellular stress and TNFα to activate the NFκB pathway is well documented (29). Previous studies have also shown that AKT1 induces activation of the NFκB by interaction with IKKα (13, 14). However, to date there are no reports addressing the potential role of AKT2 in the activation of NFκB pathway. To determine if AKT2 associates with IKKα, HEK293 cells were treated with or without TNFα, immunoprecipitated with anti-AKT2 and immunoblotted with anti-IKKα antibody or vice versa. In both instances, the association of AKT2 with IKKα was observed (Fig. 4A). Additional studies showed that AKT2-IKKα interaction was unaffected by treatment of cells with PI3K

inhibitor, wortmannin or LY294002 (Fig. 4A). These findings indicate that AKT2 constitutively associates with IKKα. In addition, we have identified putative AKT2 phosphorylation sites in the IKKα (^{18}**RERLGT**^{23}) and in NFκB inducing kinase (NIK, ^{366}**RSREPS**^{371}). To determine if IKKα and/or NIK are phosphorylated by AKT2, A2780 cells were transfected with different forms of AKT2 and treated with LY294002 and TNFα. *In vitro* AKT2 kinase assays were performed using Flag-IKKα or HA-NIK, purified from the transfected COS7 cells, as substrate. Repeated experiments show that TNFα-induced AKT2 and constitutively active AKT2 phosphorylated IKKα (Fig. 4B) but not NIK (data not shown). Phosphorylation of IKKα induced by TNFα was largely attenuated by PI3K inhibitor LY294002. Quantification analyses revealed that approximate 70% of TNFα-induced IKKα phosphorylation was inhibited by pretreatment with LY294002 (Fig. 4B). Furthermore, we assessed if AKT2 phosphorylates IKKα *in vivo*. COS7 cells were transfected with Flag-IKKα together with either constitutively active or dominant-negative AKT2 or vector alone and labeled with [γ-^{32}P]-othophosphate. IKKα immunoprecipitates prepared using anti-Flag antibody were separated by SDS-PAGE and transferred to nitrocellulose. The phospho-IKKα was detected by autoradiography. As shown in Fig. 4C, IKKα was highly phosphorylated in cells expressing constitutively active AKT2 but not in the cells transfected with pcDNA3 and dominant-negative AKT2. Collectively, these data indicate that IKKα is an AKT2 physiological substrate.

Activation of NFκB requires its dissociation from its cytosolic inhibitor, IκB, a process dependent on the phosphorylation and consequent degradation of IκB by IKK. Thus, we next examined if AKT2 induces IκB degradation. Immunobloting analyses revealed that constitutively active AKT2 significantly promoted IκBα degradation (Fig. 4D). To assess the

involvement of AKT2 in NFκB activation, HEK293 cells were co-transfected with a NFκB-luciferase reporter and either vector alone, wild-type, constitutively active or dominant negative AKT2, and treated with or without LY294002 prior to UV or TNFα stimulation. As shown in Fig. 4E, ectopic expression of wild-type AKT2 significantly enhanced UV- and TNFα-induced the NFκB activity, which was abolished by treatment of cells with LY294002 or dominant negative AKT2. Constitutively active AKT2 alone was able to induce NFκB activity to a level comparable with UV- or TNFα-treated cells transfected with wild-type AKT2. These data show that PI3K/AKT2 mediates both stress and TNFα activated NFκB pathway.

To determine AKT2 phosphorylation site of IKKα, GST fusion proteins containing either wild-type IKKα (^{18}**RERLGT**^{23}; termed GST-WT-IKKα) or mutant IKKα (^{18}**RERLGA**^{23}; termed GST-IKKαT23A) were prepared and used as substrates in *in vitro* AKT2 kinase assays. As seen in Fig. 5A, UV- and TNFα–activated AKT2, as well as constitutively active AKT2, phosphorylated GST-WT-IKKα but not GST-IKKαT23A. We next assessed the capacity of AKT2-induced IKKα to phosphorylate IκBα. Constitutively active AKT2 was expressed in HEK293 cells, and cell lysates were immunoblotted with an antibody that specifically recognizes phosphorylated IκBα at Ser^{32}. The results of these experiments show that constitutively active AKT2 increased IκBα phosphorylation approximately two-fold and that this increase was abolished by cotransfection of pcDNA3-IKKαT23A. Expression of IKKαT23A also blocked IκBα phosphorylation induced by TNFα or UV (Fig. 5B). Additional luciferase reporter experiments demonstrated that expression of IKKαT23A inhibited the TNFα– or constitutively

active AKT2-induced NFκB activation (Fig. 5C). These data indicate that phosphorylation of IKKα at Thr^{23} is required for AKT2-mediated NFκB activation.

IKKα phosphorylation by AKT2 Is Required for Inhibition of JNK but not p38 Activation – Recent studies showed that NFκB exerts its cell survival function by inhibition of JNK activation in response to extracellular stress (30, 31). However, it is currently unknown whether Akt-induced NFκB activation results in inhibition of JNK. Therefore, we next examined if AKT2-activated IKKα is required for AKT2 inhibition of JNK and p38 activities induced by stress and TNFα. The activation of JNK and p38 was examined in HEK293 cells transfected with IKKα or IKKαT23A together with or without constitutively active AKT2. Western blotting analyses with phospho-JNK and -p38 antibodies revealed that wild type IKKα did not significantly enhance AKT2 inhibition of JNK (Fig. 6). However, expression of IKKαT23A abrogated the effects of constitutively active AKT2 on inhibition of JNK (Fig. 6). Similar to the results shown in Fig. 3, TNFα-induced JNK activation reached the maximal level at 10 min of the stimulation, which was neither significantly inhibited by constitutively active AKT2 nor affected by expression of IKKαT23A (Fig. 6). Therefore, these data indicate that inhibition of JNK activation by AKT2/NFκB could be via a mechanism of induction of dephosphorylation of JNK by AKT2/IKKα/NFκB cascade.

AKT2 Activation Inhibits Stress-induced Apoptosis – It has been documented that various stresses and TNFα are capable of inducing apoptosis in different cell types through activation of JNK and p38 pathways (29). As PI3K/Akt is essential for cell survival and activated AKT2

inhibits JNK/p38 and induces NFκB pathway, we investigated the role of PI3K/AKT2 in stress- and TNFα-induced programmed cell death. AKT2 stably-transfected A2780 cells were pretreated with or without LY294002 for 2 h prior to exposure to UV, heat shock, NaCl, or TNFα. As determined by the TUNEL assay, inhibition of PI3K activity dramatically increased the percentage of cells undergoing apoptosis in response to UV or TNFα (Fig. 7). Moreover, inhibition of AKT2 activity by expression of dominant-negative AKT2 increased the percentage of apoptotic cells in the UV- and TNFα-treated populations by approximately 1-fold. On the other hand, cells expressing constitutively active AKT2 were resistant to UV- and TNFα-induced apoptosis. These data show that the PI3K/AKT2 pathway plays a key role in protecting cells from apoptosis induced by extracellular stress or TNFα.

DISCUSSION

In this report, we have provided evidence that AKT2 is activated by extracellular stress and TNFα through a PI3K-dependent pathway in human epithelial cells. Most importantly, the activation of AKT2 inhibits stress- and TNFα-induced JNK and p38 activities and activates the NFκB cascade, leading to protection of cells from stress- and TNFα-induced apoptosis.

Previous studies have shown that stress activates cell membrane receptors, including those for EGF, PDGF, and IGF. As a result, receptors associate with numerous proteins that activate downstream signaling molecules (1-3). One such protein is PI3K, which has been implicated in the regulation of nearly all stress signaling pathways (1). Since the AKTs are major downstream targets of PI3K, their role in the stress response has been recently investigated. In Swiss 3T3 cells, both oxidative stress and heat shock were shown to induce a

marked activation of AKT1 and AKT3 but not AKT2 (19). AKT1 activation by hyperosmotic stress in COS7 and NIH 3T3 cells has also been demonstrated (17). In this study, we show that AKT2 is activated by different stress conditions including UV irradiation, hyperosmolarity and heat shock, as well as by TNFα, in several human epithelial cell lines.

Three isoforms of Akt display high sequence homology and share similar upstream regulators and downstream targets as identified so far. However, there are clear differences between them in terms of biological and physiological function. In addition to the more prominent role of AKT2 in human malignancy and transformation (7, 32), the expression patterns of AKT1, AKT2, and AKT3 in normal adult tissues as well as during development are quite different (4, 8, 33). Recent studies suggest that AKT1, AKT2, and AKT3 may interact with different proteins and thus may play different roles in signal transduction. For instance, the Tcl1 oncoprotein preferentially binds to and activates AKT1 but not AKT2 (34). Gene knockout studies revealed that Akt1-deficient mice display defects in both fetal and postnatal growth but, unlike $Akt2^{-/-}$ mice, do not exhibit a type II diabetic phenotype; these differences suggest that the functions of AKT1 and AKT2 are non-redundant with respect to organismal growth and insulin-regulated glucose metabolism (21-23). It has been also shown that AKT2 but not AKT1 plays a specific role in muscle differentiation (35, 36). In this study, we demonstrated that AKT2 is activated by a variety of stress conditions in human epithelial cells but not in fibroblasts, suggesting that activation of different isoforms of Akt is cell type specific in response to extracellular stress.

It is controversial whether stress-induced AKT1 activation is mediated by the PI3K pathway (17-19). Two previous reports showed that PI3K inhibitors did not block heat shock- or H_2O_2-induced activation of AKT1 and thus suggested that stress (unlike growth factors) activates AKT1 in a PI3K-indpendent manner (17, 18). However, opposite results were observed by other groups (19, 20). Konishi *et al.* also provided evidence of AKT1 activation by H_2O_2 and heat shock through both PI3K- dependent and -independent pathways (18). We previously demonstrated that activation of AKT2 by growth factors required PI3K activity whereas both PI3K-dependent and -independent pathways contributed to AKT2 activation by Ras (26). In this report, we show that PI3K inhibitors completely block AKT2 activation induced by UV-C, heat shock, and hyperosmolarity, indicating that stress activates AKT2 via the PI3K pathway.

JNK and p38 are stress MAP kinases that are activated by cytokines and a variety of cellular stresses (28). Like the classical MAP kinase kinase (MEK), direct activators for JNK and p38 have been identified. JNK is activated by phosphorylation of tyrosine and threonine by the dual specificity kinases, MKK4/SEK1 and MKK7. Similarly, p38 is activated by MKK3 and MKK6. However, biochemical studies have documented the existence of other JNK and p38 activators or inhibitors in cells stimulated by a variety of cellular stresses (28). Although previous reports showed that Akt, JNK, and p38 are downstream targets of PI3K and represent parallel pathways in response to stress (17-20, 37, 38), the data presented in this study indicate that stress- and TNFα-induced activation of AKT2 inhibits the JNK and p38 activities, suggesting that AKT2 cross-talks with JNK and p38 stress pathways.

NFκB is another critical stress response pathway (29). Activation of NFκB is achieved through the signal-induced proteolytic degradation of IκB, which is associated with and inhibits the activity of NFκB in the cytoplasm. The critical event that initiates IκB degradation is the stimulus-dependent activation of the IκB kinases, IKKα and IKKβ, which phosphorylate IκB at specific N-terminal serine residues (Ser^{32} and Ser^{36} for IκBα; Ser^{19} and Ser^{23} for IκBβ). Phosphorylated IκB is then selectively ubiquitinated by an E3 ubiquitin ligase and degraded by the 26S proteasome, thereby releasing NFκB for translocation to the nucleus where it initiates the transcription of target genes (29). Moreover, two MAP kinase kinase kinase (MAPKKK) members, NIK and MEKK-1, have been reported to enhance the activity of the IKKs, and consequently trigger the phosphorylation and destruction of the IκBs and induce the activition of the NFκB pathway (29). Recent studies also showed that AKT1 induces the NFκB cascade through activation of IKK and degradation of IκB (13, 14). In this report, we show that AKT2 physically binds to and phosphorylates IKKα, but not NIK even though NIK contains an AKT2 phosphorylation consensus sequence. When activated by stress or TNFα, AKT2 degrades IκB and activates NFκB-mediated transcription, indicating that stress-activated AKT2 targets the NFκB pathway.

Importantly, we have provided evidence that activation of AKT2 induced by stress and TNFα inhibits JNK activity through activation of the NFκB pathway to protect cells from apoptosis in response to these stimuli. Previous studies showed that the AKT2 pathway is important for cell survival and malignant transformation (7, 24, 32). The data presented here show that cells expressing constitutively active AKT2 are resistant to stress- and TNFα-induced

apoptosis and that dominant-negative AKT2 and LY294002 sensitize cells to stress- and TNFα-induced programmed cell death. These findings indicate that stress-induced AKT2 activation promotes cell survival. Among the stress-activated kinases are JNK, recent studies demonstrated that activation of JNK and p38 plays an important role in triggering apoptosis in response to extracellular stress and TNFα (39-42), whereas activation of NFκB protects cells from programmed cell death (29). Although a number of downstream targets of AKT2 have been identified, our data indicate that AKT2-inhibited JNK and p38 activities and AKT2-induced NFκB activation could play, at least in part, an important role in the AKT2 pathway that protects cells from stress- and TNFα-induced apoptosis. Recent reports demonstrated that NFκB-upregulated Gadd45β and Xiap inhibited JNK activation and abrogated TNFα-induced programmed cell death (30, 31). Our cDNA microarray experiments showed that constitutively active AKT2 induces *Xiap* (43). Thus, AKT2 inhibition of JNK activity could be due to upregulation of Xiap by NFκB pathway (Fig. 8). Further studies are required to characterize the mechanism of inhibition of p38 stress pathway by AKT2 and involvement of Xiap in AKT2/NFκB inhibition of the JNK activation.

Acknowledgements - We are grateful to Michael Karin for HA-JNK1 construct and Roger J. Davis for the GST-c-Jun (1-79) and pCMV-Flag-p38. We are also grateful to the DNA Sequence Facility at the H. Lee Moffitt Cancer Center for sequencing expression constructs.

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Figure Legends

Fig. 1. **AKT2 is activated by cellular stress and TNFα.** *A*, *In vitro* kinase assay of AKT2 immunoprecipitates prepared from A2780 cells transiently transfected with HA-AKT2. Cells were exposed to 100 ng/ml IGF-1 (15 min), heat shock (45^0C for 20 min), 0.4 M NaCl (15 min), 40 J/m^2 UV-C (254 nm), or TNFα 20 ng/ml (15 min) and AKT2 activity was determined by *in vitro* kinase assay using histone H2B as substrate. *B*, OVCAR3 cells were treated with indicated stimuli and immunoprecipitated with anti-AKT2 antibody. The immunoprecipitates were subjected to *in vitro* kinase assay (upper) and Western blotting analyses with anti-phospho-Ser473 Akt (middle) or anti-AKT2 (lower) antibody. Bottom panel shows relative AKT2 kinase activity quantified by Phosphoimager. Each experiment was repeated three times.

Fig. 2. **Activation of AKT2 by cellular stress and TNFα is PI3K-dependent.** *A, In vitro* PI3K assay. HA-AKT2-transfected HEK293 cells were exposed to the indicated stimuli. Upper panel: PI3K immunoprecipitates were prepared with anti-pan-p85 antibody and assayed for PI3K activity; Middle panel shows p85 protein level using anti-p85 antibody and bottom panel represents relative PI3K activity quantified by Phosphoimager. *B*, HA-AKT2-transfected A2780 cells were treated with LY294002 for 30 min prior to exposure to indicated stimuli. HA-AKT2 immunoprecipitates were subjected to *in vitro* kinase assay. Results were confirmed by four independent experiments.

Fig. 3. **AKT2 kinase inhibits UV- and TNFα-induced JNK activation.** *A*, Western blotting analyses of HEK293 cells transfected with indicated plasmids. Cells were lysed at indicated times after incubation with TNFα and analyzed with anti-phospho-JNK (upper), -total JNK

(middle) and –HA (lower) antibodies. The immunoblotting analyses were repeated three times. *B,* The procedures are the same as (*A*), except the membranes were probed with anti-phopho-p38 (upper), -total p38 (middle) and –HA (lower). Graphical presentations show the normalized density of phosphorylated JNK (bottom of panel A) and p38 (bottom of panel B), decaying from 100 percent.

Fig. 4. **AKT2 interacts with and phosphorylates IKKα, leading to IκBα degradation and NFκB activation.** *A*, *Left panel,* Western blotting analyses. HEK293 cell lysates were immunoprecipitated with anti-AKT2 or IgG (control) and detected with anti-IKKα (top) or anti-AKT2 (bottom) antibody. *Right panel*, HEK293 cells were treated with LY294002 or wortmannin for 30 min; followed by TNFα for 20 min. Immunopreciptates were prepared with anti-IKKα antibody or IgG and immunoblotted with antibody to AKT2 (top) or IKKα (bottom). *B*, *In vitro* kinase assay analyses of immunoprecipitates prepared from A2780 cells transfected with indicated plasmids, using immunopurified Flag-IKKα as substrate (upper). Expression of Flag-IKKα was confirmed by immunoblotting analysis with anti-Flag antibody (middle). Bottom panel shows relative phosphorylation levels of IKKα by AKT2. *C*, *In vivo* labeling of IKKα from COS7 cells transfected with indicated DNA constructs, treated with or without TNFα, and incubated with [γ-^{32}P]-othophosphate for 4 h. IKKα immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, exposed to film (top), and then detected with anti-IKKα antibody (bottom). *D,* AKT2 induces IκBα degradation. HEK293 cells were transfected with indicated plasmids and treated with cycloheximide (50 μg/ml) for 1 hour before treatment with 50 ng/ml TNFα for up to 60 min. Cell lysates were immunoblotted with antibody to IκBα (left panels) or β–actin (right panels). Degradation of IκBα was quantified with a

densitometer. Bottom panel shows the degradation rate of IκBα by normalizing density of IκBα bands at 0 time point as 100 percent. *E,* Reporter assays. HEK293 cells were transfected with 2xNFκB-Luc, β-galactosidase and WT-AKT2, Myr-AKT2, or DN-AKT2, pretreated with or without LY294002 and subsequently exposed to 40 J/m^2 UV-C or 20 ng/ml TNFα. Cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. Error bars represent standard deviation. Data were obtained from triplicate experiments.

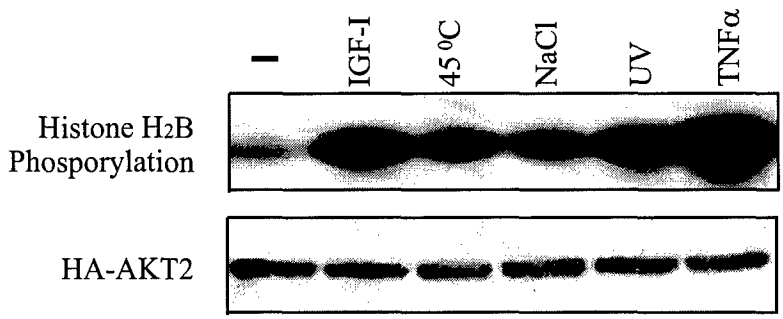
Fig. 5. **AKT2-phosphorylated IKKα at threonine-23 is required for stress-induced NFκB.** *A,* AKT2 phosphorylation of IKKα at threonine-23. *In vitro* kinase assay of AKT2 immunoprecipitates prepared from A2780 cells transfected with indicated plasmids and treated with or without UV or TNFα. GST-fused wild-type IKKα (^{18}**RERLGT**^{23}) or mutant IKKα (^{18}**RERLGA**^{23}) was used as substrate. *B,* AKT2 induces IκBα phosphorylation. HEK293 cells were transfected with indicated expression constructs. Thirty-six hours after transfection, cells were treated with 20 ng/ml TNFα for 30 min or irradiated with 40 J/m^2 UV followed by 30 min incubation. Cell lysates were immunoblotted with anti-phospho-IκBα (upper) or anti-IκBα (middle) antibody. The band density of the phospho-IκBα was quantified (bottom). *C,* Luciferase reporter assay. HEK293 cells were transfected with indicated plasmids. After treatment with or without 20 ng/ml TNFα for 12 h, cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. Results were obtained from three independent experiments.

Fig. 6. **AKT2 phosphorylation of IKKα is required for its inhibition of TNFα-induced JNK activity.** Immunoblotting analyses of HEK293 cells transfected with indicated expression constructs and treated with TNFα (20 ng/ml). The blots were probed with anti-phospho-JNK (upper) and –total JNK (middle) antibodies. Results represent one of three independent experiments. Bottom panel shows the quantification of phosphorylated JNK at indicated time points.

Fig. 7. **AKT2 activation inhibits stress-induced apoptosis.** A2780 cells, stably transfected with constitutively active AKT2, DN-AKT2 or vector alone, were pretreated with or without 25 μM LY294002 for 2 h prior to exposure to UV-C (300 J/m^2) (*A*), or TNFα (50 ng/ml) for 24 h (*B*). Apoptosis was assessed by TUNEL assay. *Lower panel:* Quantitation of data shown in (*A*) and (*B*) were derived from triplicate experiments. Error bars represent standard deviation.

Fig. 8. **Schematic illustration of negative regulation of JNK by AKT2/NFκB.**

A


–
IGF-I
45 °C
NaCl
UV
TNFα
Histone H2B Phosporylation
HA-AKT2

B

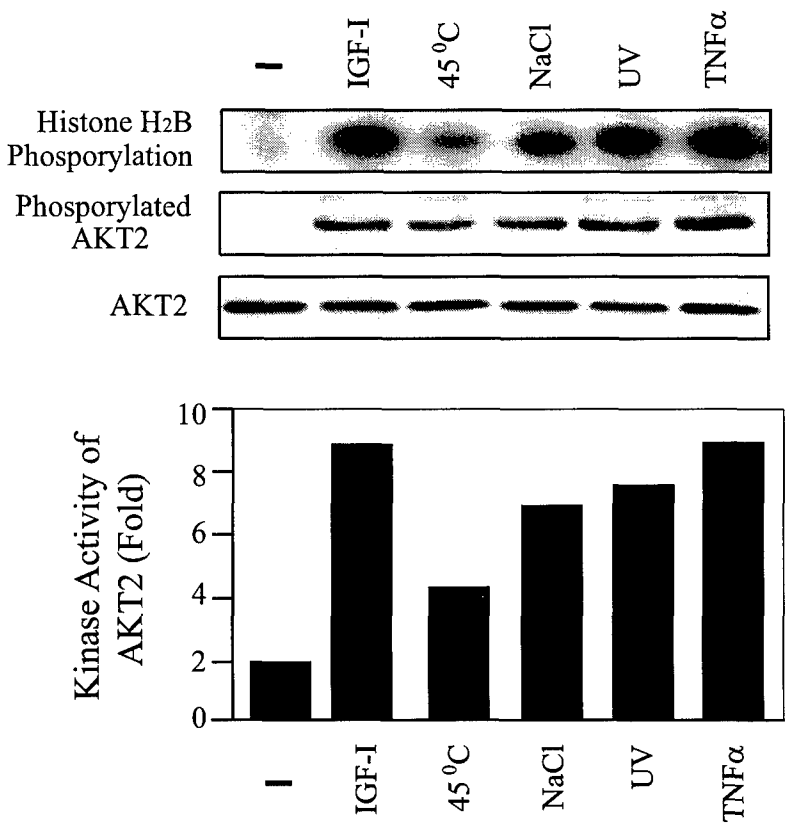

–
IGF-I
45 °C
NaCl
UV
TNFα
Histone H2B Phosporylation
Phosporylated AKT2
AKT2
Kinase Activity of AKT2 (Fold)
10
8
6
4
2
0

FIG 1

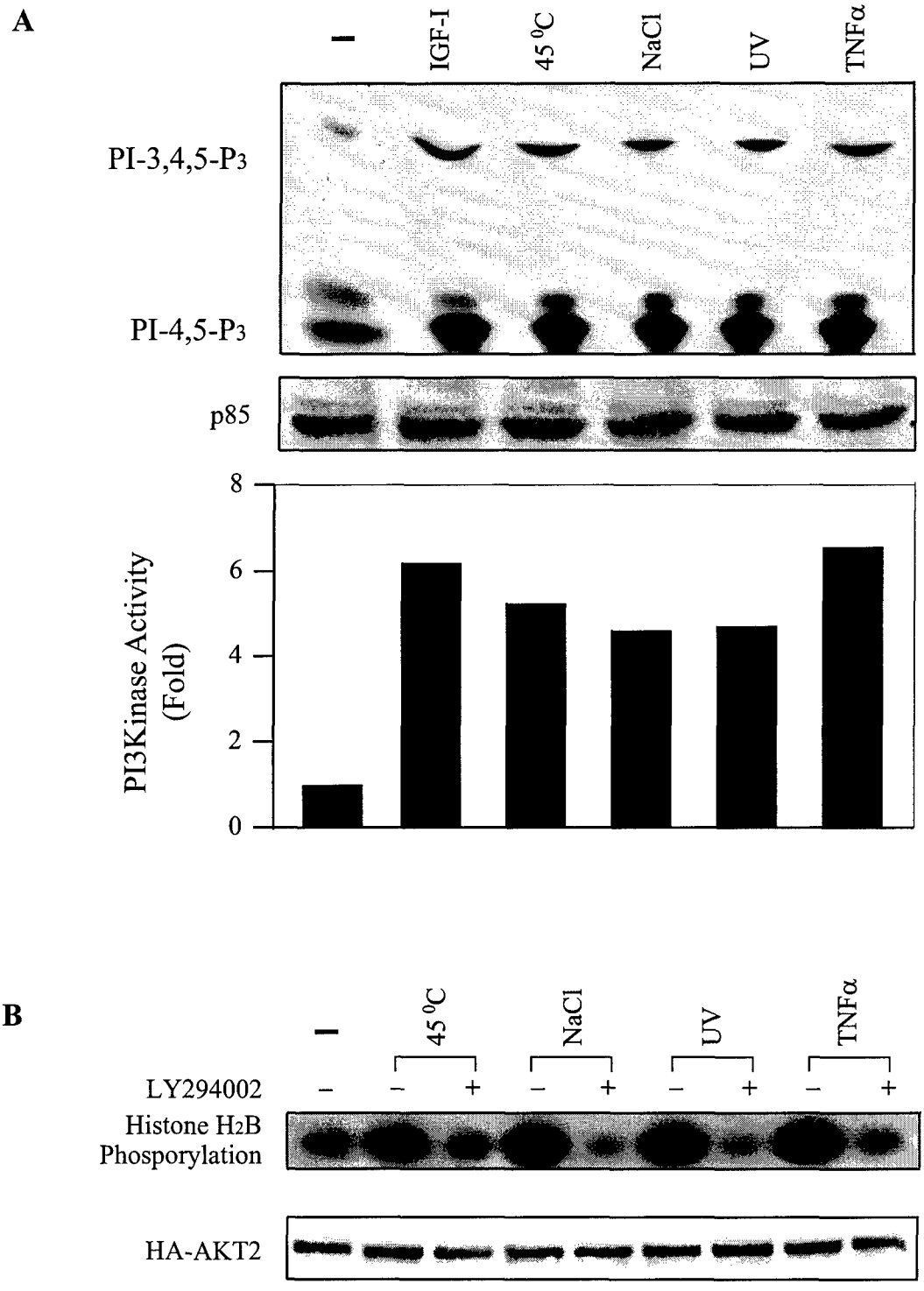
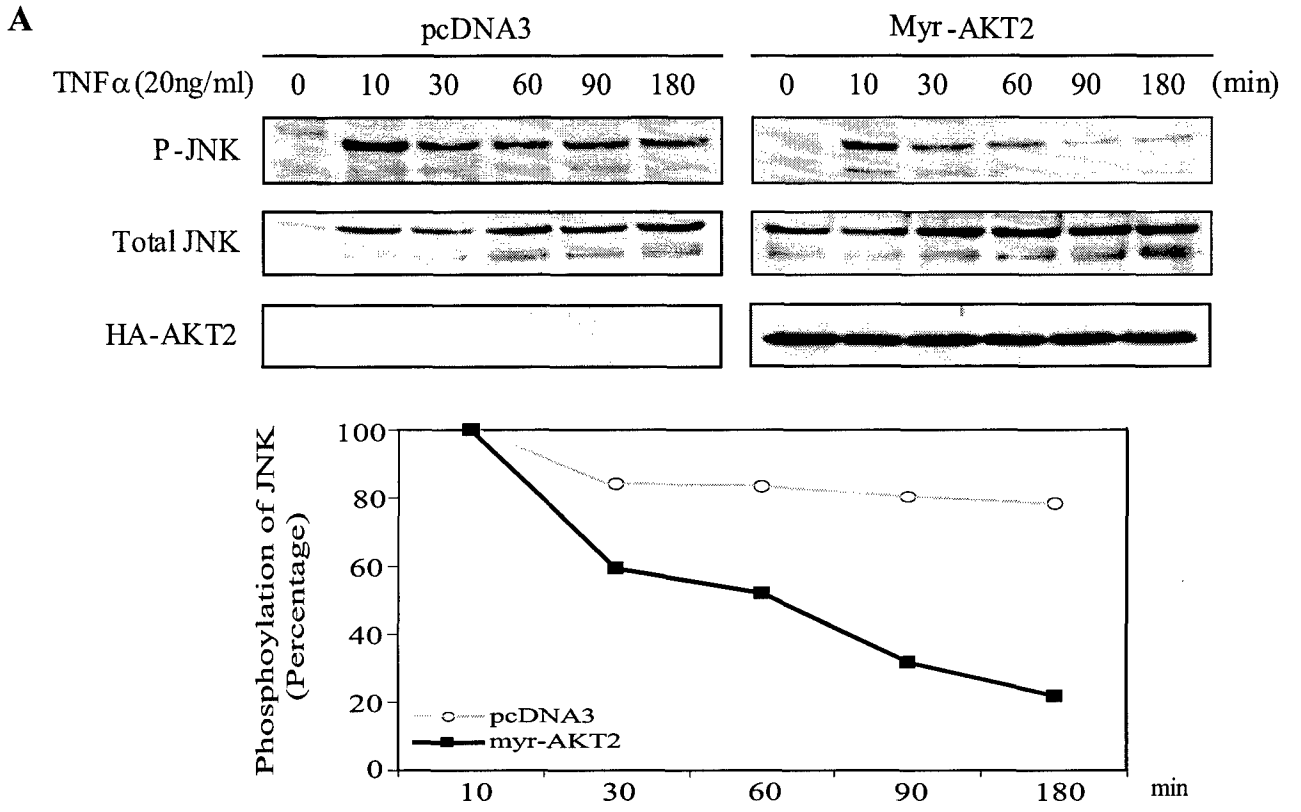

A
–
IGF-I
45 °C
NaCl
UV
TNFα
PI-3,4,5-P3
PI-4,5-P3
p85
PI3Kinase Activity (Fold)
8
6
4
2
0
B
–
45 °C
NaCl
UV
TNFα
LY294002
– – + – + – + – +
Histone H2B Phosporylation
HA-AKT2

FIG 2


A
pcDNA3
Myr-AKT2
TNFα(20ng/ml) 0 10 30 60 90 180 0 10 30 60 90 180 (min)
P-JNK
Total JNK
HA-AKT2
Phosphoylation of JNK (Percentage)
100
80
60
40
20
0
pcDNA3
myr-AKT2
10 30 60 90 180 min

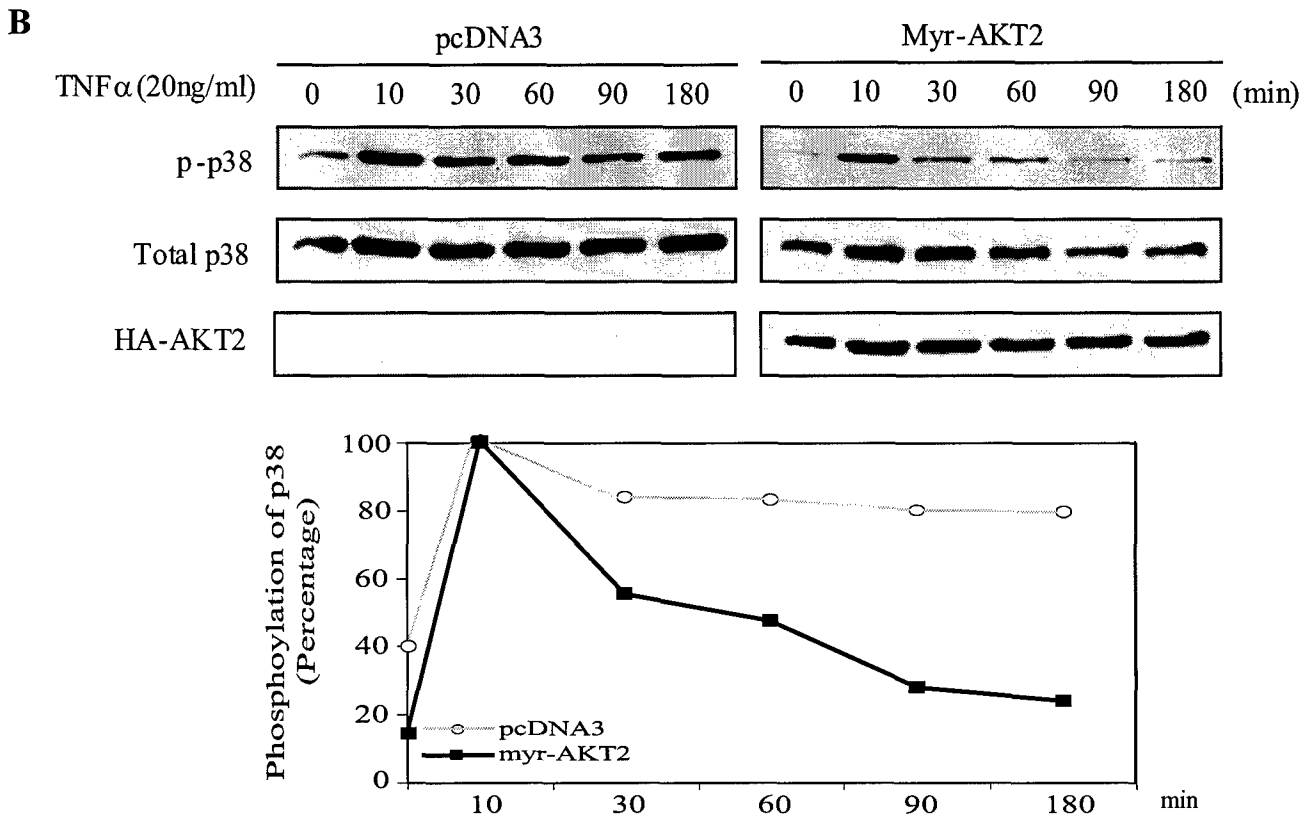
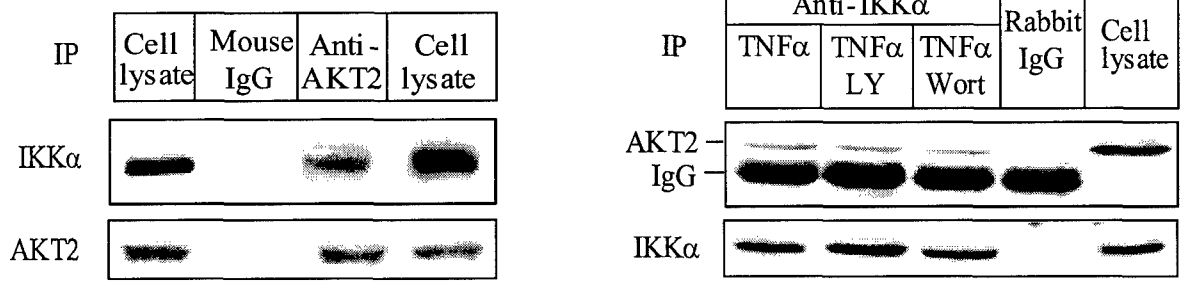
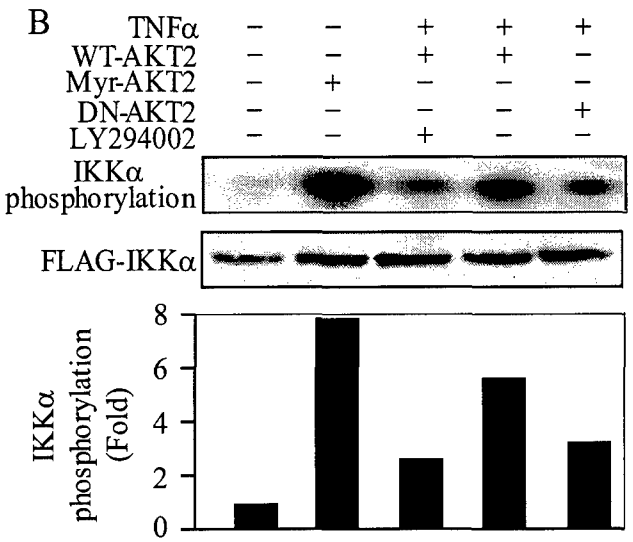

B
pcDNA3
Myr-AKT2
TNFα(20ng/ml) 0 10 30 60 90 180 0 10 30 60 90 180 (min)
p-p38
Total p38
HA-AKT2
Phosphoylation of p38 (Percentage)
100
80
60
40
20
0
pcDNA3
myr-AKT2
10 30 60 90 180 min

FIG 3

A


IP
Cell lysate
Mouse IgG
Anti-AKT2
Cell lysate
IKKα
AKT2
IP
Anti-IKKα
TNFα
TNFα LY
TNFα Wort
Rabbit IgG
Cell lysate
AKT2
IgG
IKKα

B


TNFα
WT-AKT2
Myr-AKT2
DN-AKT2
LY294002
IKKα phosphorylation
FLAG-IKKα
IKKα phosphorylation (Fold)
8
6
4
2
0

C

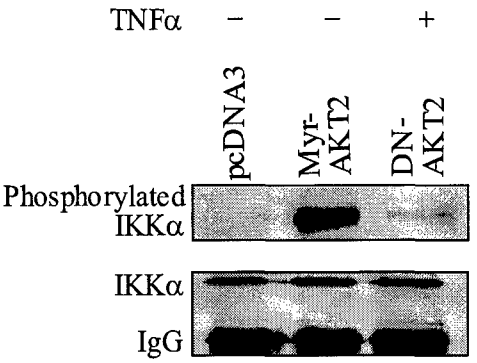
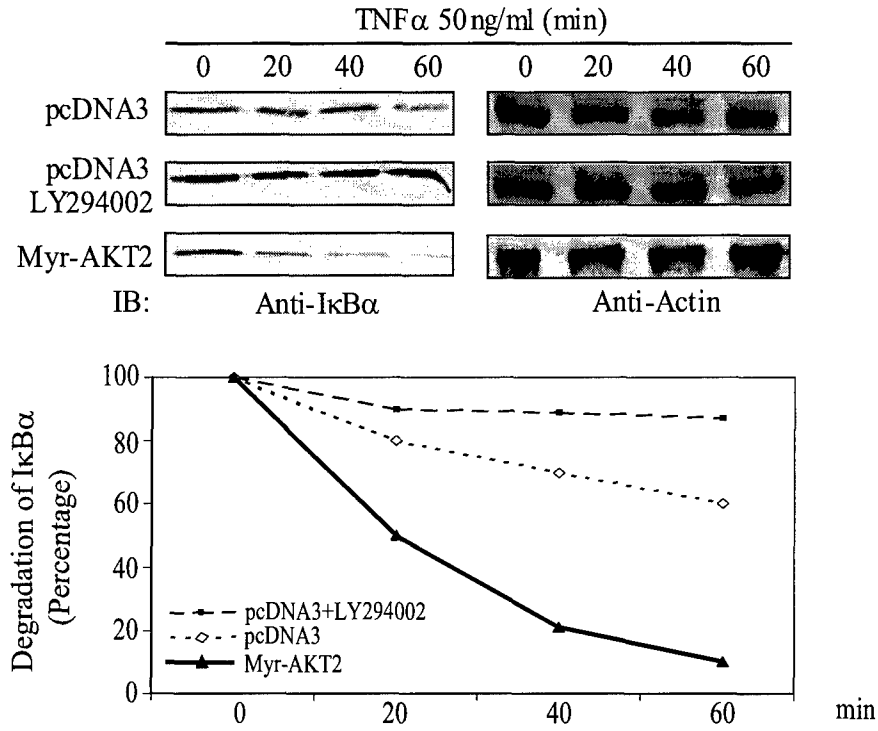

TNFα
pcDNA3
Myr-AKT2
DN-AKT2
Phosphorylated IKKα
IKKα
IgG

FIG 4

D



E

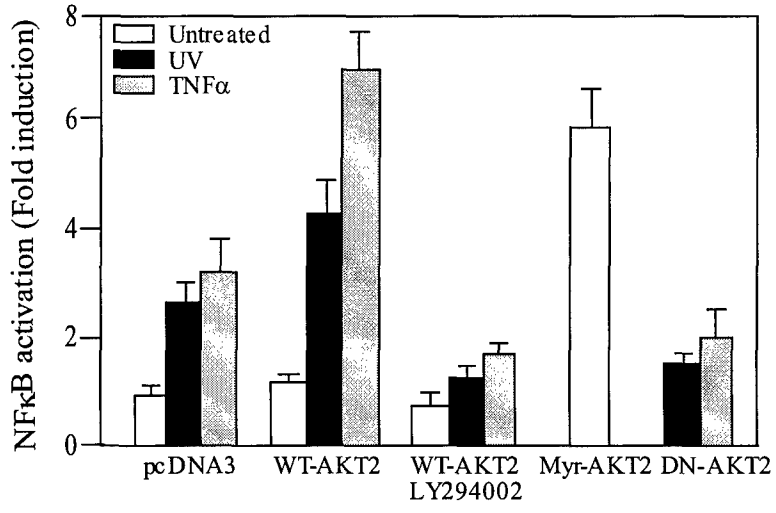


FIG 4 (Continued)

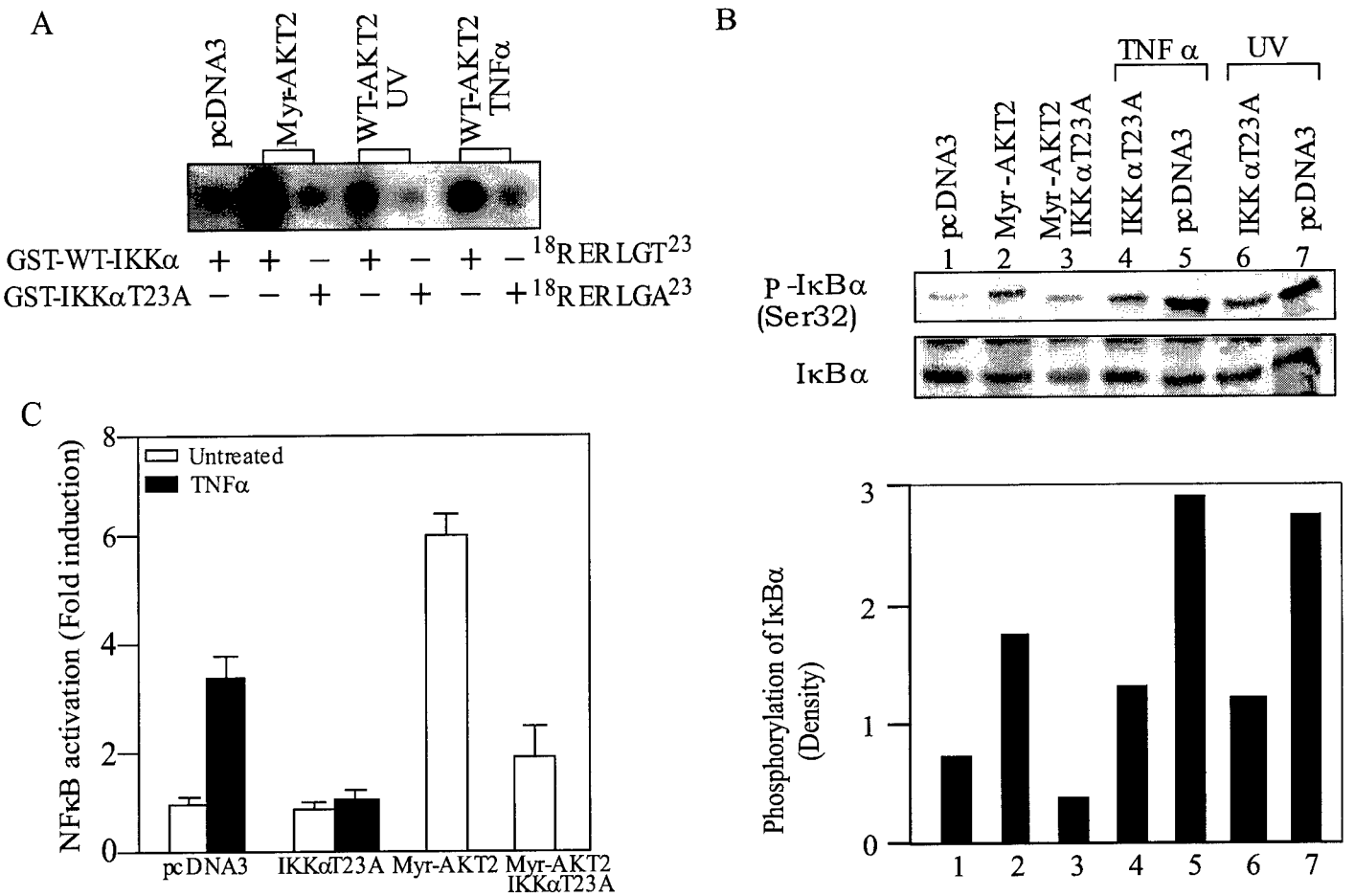
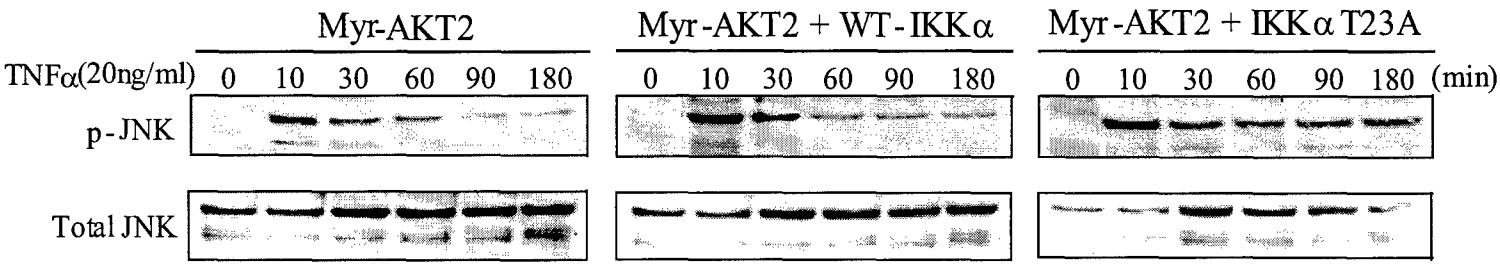

A
pcDNA3
Myr-AKT2
WT-AKT2 UV
WT-AKT2 TNFα
GST-WT-IKKα + + − + − + − 18RERLGT23
GST-IKKαT23A − − + − + − + 18RERLGA23
B
TNF α
UV
pcDNA3
Myr-AKT2
Myr-AKT2 IKKαT23A
IKKαT23A
pcDNA3
IKKαT23A
pcDNA3
1 2 3 4 5 6 7
P-IκBα (Ser32)
IκBα
C
NFκB activation (Fold induction)
Untreated
TNFα
pcDNA3
IKKαT23A
Myr-AKT2
Myr-AKT2 IKKαT23A
Phosphorylation of IκBα (Density)

FIG 5


Myr-AKT2
Myr-AKT2 + WT-IKKα
Myr-AKT2 + IKKα T23A
TNFα(20ng/ml) 0 10 30 60 90 180 0 10 30 60 90 180 0 10 30 60 90 180 (min)
p-JNK
Total JNK

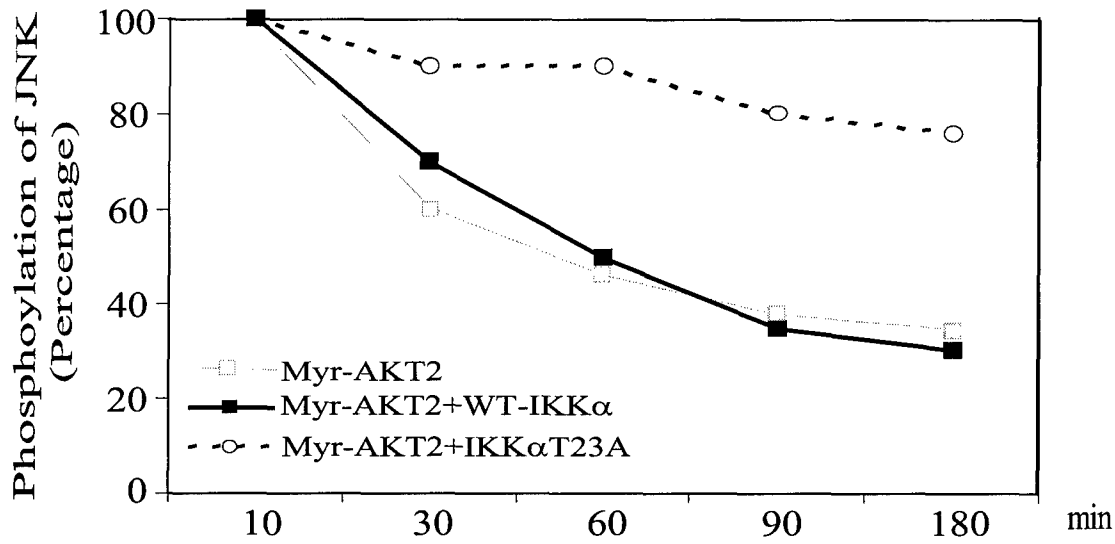
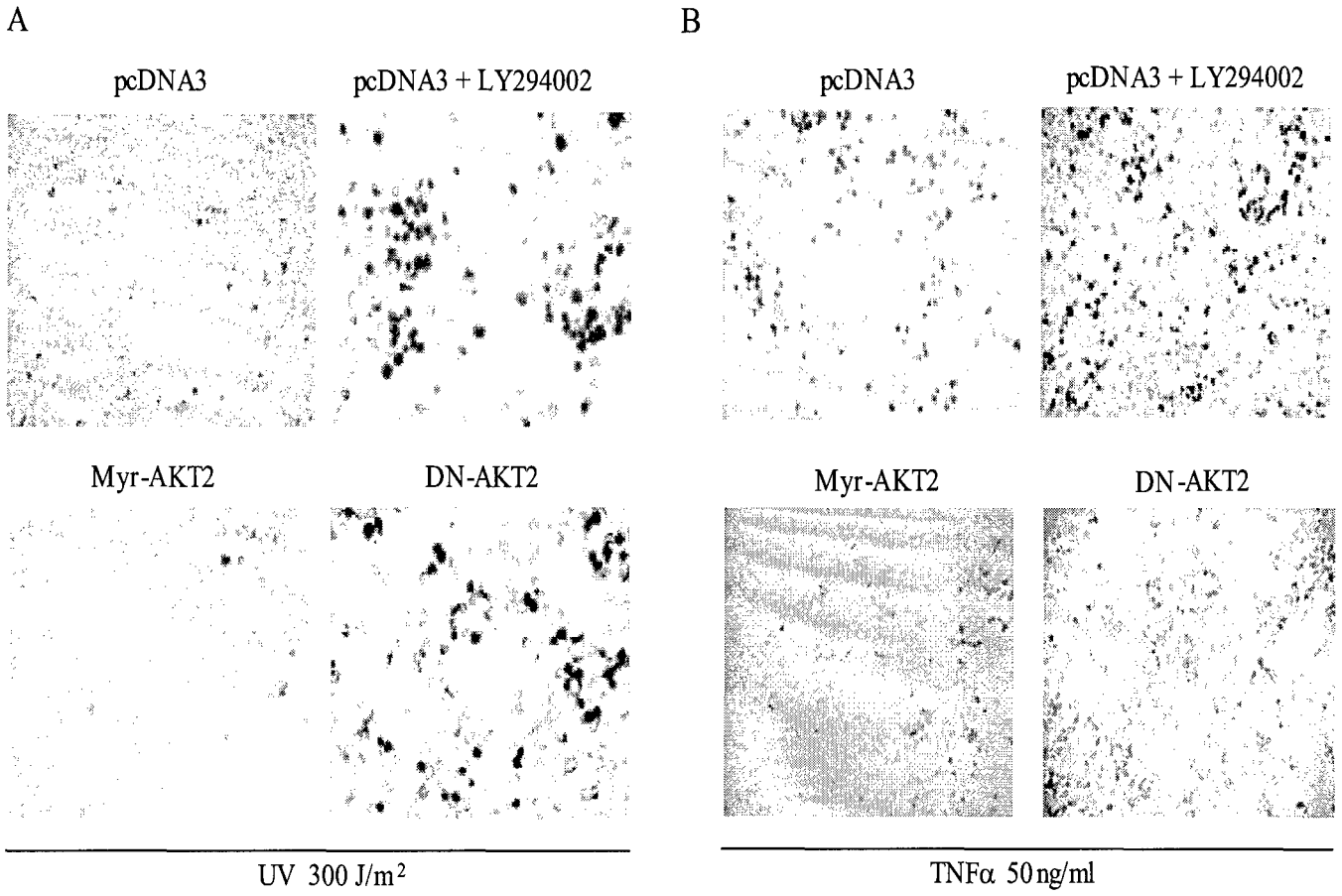

Phosphoylation of JNK (Percentage)
100
80
60
40
20
0
10
30
60
90
180
min
Myr-AKT2
Myr-AKT2+WT-IKKα
Myr-AKT2+IKKαT23A

FIG 6


A
pcDNA3
pcDNA3 + LY294002
Myr-AKT2
DN-AKT2
UV 300 J/m2
B
pcDNA3
pcDNA3 + LY294002
Myr-AKT2
DN-AKT2
TNFα 50ng/ml

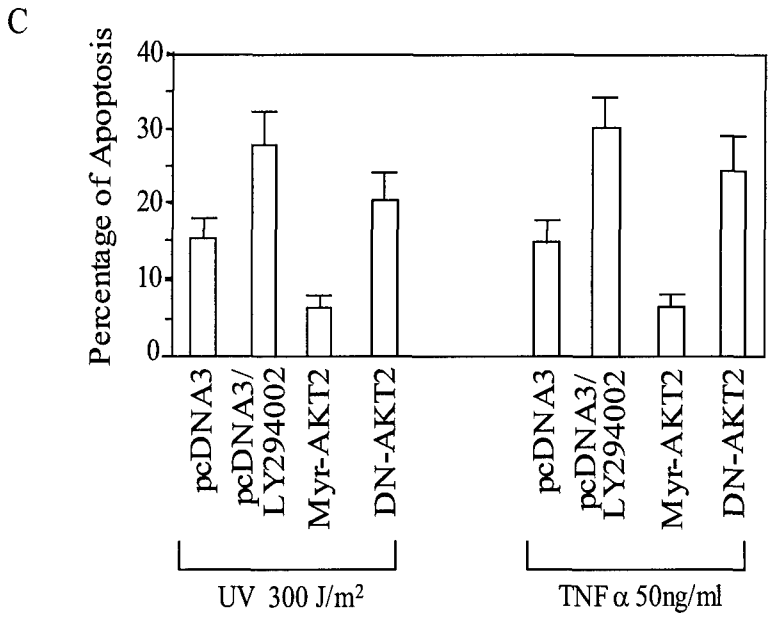

C
Percentage of Apoptosis
40
30
20
10
0
pcDNA3
pcDNA3/ LY294002
Myr-AKT2
DN-AKT2
pcDNA3
pcDNA3/ LY294002
Myr-AKT2
DN-AKT2
UV 300 J/m2
TNF α 50ng/ml

FIG 7

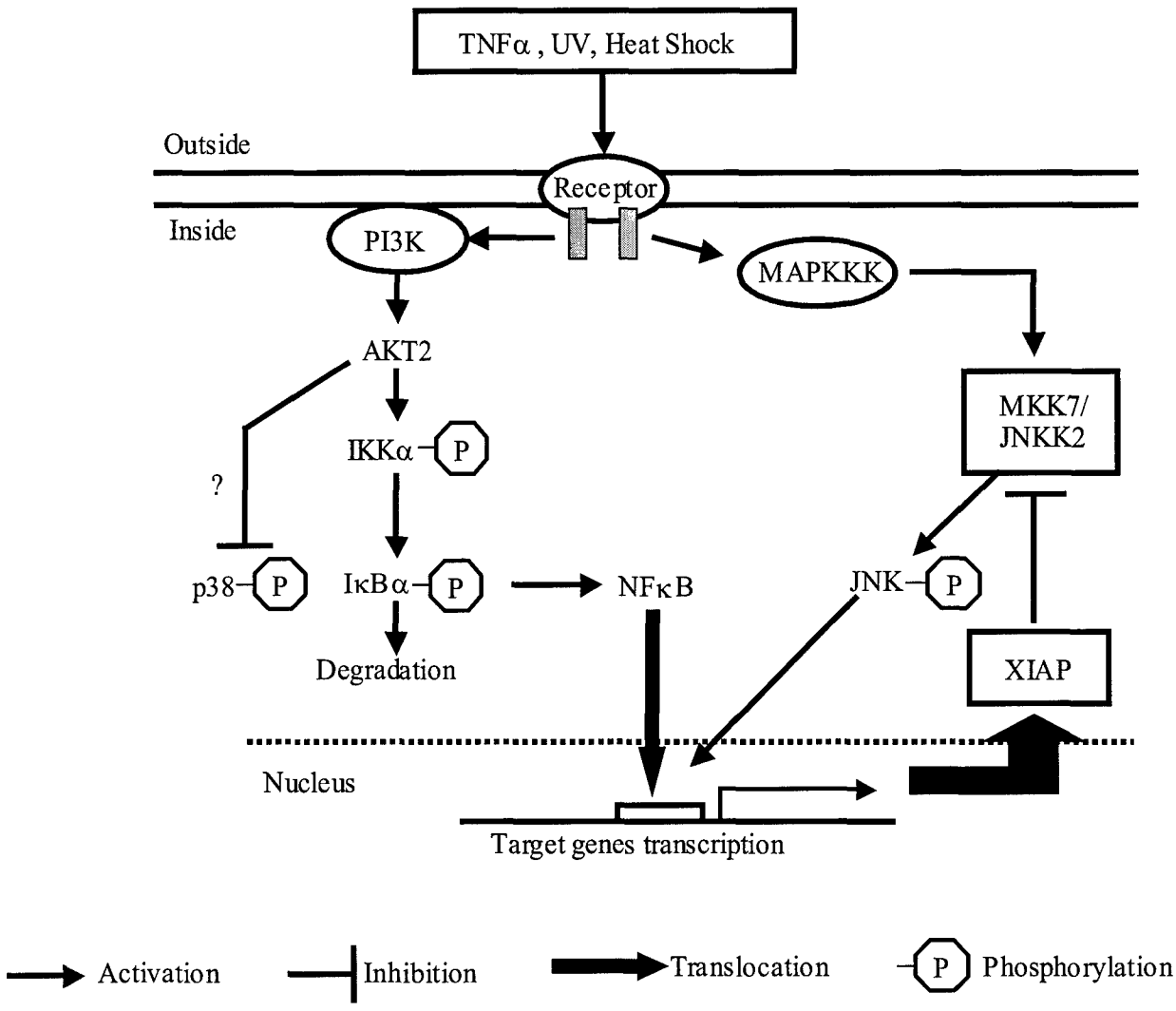

TNFα , UV, Heat Shock
Outside
Receptor
Inside
PI3K
MAPKKK
AKT2
MKK7/
JNKK2
IKKα
P
?
p38
P
IκBα
P
NFκB
JNK
P
Degradation
XIAP
Nucleus
Target genes transcription
Activation
Inhibition
Translocation
P
Phosphorylation

FIG 8

Zeng-qiang Yuan, Predoctoral Fellowship Awardee

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Zeng Qiang YUAN	Ph. D. Candidate

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

INSTITUTION AND LOCATION	DEGREE (*if applicable*)	YEAR(s)	FIELD OF STUDY
Qingdao Medical College, Qingdao, P. R. China	M.D.	1995	Medicine
Beijing Academic College, Beijing, P. R. China	M.S.	1998	Pathophysiology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Professional Experience:

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Honors and Awards:

1991-1995 Outstanding Student Scholarship, Qingdao Medical College, Qingdao, P. R. China
1996-1998 Outstanding Student Scholarship, Department of Pathophysiology, Basic Research Institute, Beijing Academic College, Beijing, P. R. China
2000 Superior Presenter in USF HSC Research Day in February 10, 2000.
Travel Award for AACR meeting from Medical School, USF
2001 Fellowship from Department of Defense Breast Cancer Research Program (BCRP)
Superior Presenter in USF HSC Research Day in February 10, 2001.
2002 Superior Presenter in USF HSC Research Day in February 14, 2002.

Publications:

1. **Yuan, Z.Q.**, Sun, M., Feldman, R.I., Wang, G., Ma, X., Coppola, D., Nicosia, S.V. and Cheng, J.Q. Frequent alterations of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. Oncogene 19:2324-2330, 2000.

2. **Yuan ZQ**, Feldman RI, Sun M, Olashaw NE, Coppola D, Sussman GE, Shelley SA, Nicosia SV, Cheng JQ. Inhibition of JNK by cellular stress- and TNFa-induced AKT2 through activation of the NFkB pathway in human epithelial cells. J Biol Chem 2002 Jun 4; [epub ahead of print]

3. Sun M, Wang G, Paciga JE, Feldman RI, **Yuan ZQ**, Ma XL, Shelley SA, Jove R, Tsichlis PN, Nicosia SV, Cheng JQ. AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. Am J Pathol 2001 Aug; 159(2): 431-7

4. Sun M, Paciga JE, Feldman RI, **Yuan ZQ**, Coppola D, Lu YY, Shelley SA, Nicosia SV, Cheng JQ. Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. Cancer Res 2001 Aug 15; 61(16): 5985-91